LIPID PHASE TRANSITIONS CONTROL β-HYDROXYBUTYRATE DEHYDROGENASE ACTIVITY IN DEFINED-LIPID PROTEIN COMPLEXES

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1. Introduction

The purified lipid-free apo-enzyme of β-hydroxybutyrate dehydrogenase (BDH) specifically requires lecithin to bind the NADH coenzyme [1]. When the soluble apo-enzyme is reactivated by mixing with aqueous dispersions of lecithin vesicles, the activity of the complexes is not inhibited below the phase transition temperature of the lipid [1], in contrast to many other membrane proteins. This suggests that when the apo-enzyme is reactivated by this procedure, it may interact primarily with the choline headgroups of the lipid bilayer. However, it is not clear that this interaction necessarily resembles that of BDH with lecithin in vivo. The conformation of the soluble apo-enzyme may be so altered during preparation that it can no longer interact with the lecithin chains; alternatively, the apo-enzyme may be unable to penetrate the hydrophobic chain region of a preformed lipid vesicle to achieve a normal in vivo interaction. A critical test is whether the enzyme would respond to the phase transition when it interacts with lecithin in the same way as in vivo. Here we have used two techniques to exchange the lipids around BDH in the mitochondrial membrane with defined synthetic lecithins. Both techniques avoid exposure of the lipid binding sites on BDH to the aqueous environment, and the activity of the enzyme is maintained throughout the lipid exchange process. These experiments confirm earlier reports [1,2] that a choline headgroup on the phospholipid is essential for activity and further show that other lipid classes present in the native membrane, including cholesterol, can completely inhibit activity. However, in complexes with defined lecithins the activity of BDH is also critically dependent on the physical state of the acyl chains: when the associated lipid is in the crystalline state the activity of the enzyme is abolished. We consider that the lipid–protein interactions in these complexes closely resemble those occurring in vivo, and that BDH activity is determined by both the polar headgroup and the chain conformation of the associated lipids. We also present preliminary evidence that BDH interacts selectively with dioleoyl lecithin (DOL) in the presence of other mitochondrial lipids, which probably implies lateral segregation of lipids imposed by the protein.

2. Materials and methods

Ox heart mitochondria were prepared by the method of Smith [3] and the pellet suspended in a sucrose buffer containing 0.25 M sucrose, 1.0 M KCl, 0.05 M potassium phosphate (pH 8.0), at a protein concentration of 50 mg/ml.

Ninety per cent of the endogenous mitochondrial lipids were replaced by dioleoyl lecithin (DOL) or dimyristoyl lecithin (DML) by the technique of lipid substitution, described in detail elsewhere [4]. The endogenous mitochondrial lipid pool was equilibrated with an excess of the defined exogenous lipid in the presence of cholate, and the mitochondrial pellet with the substituted lipid was recovered from a sucrose gradient after centrifugation. The excess lipid was retained in the supernatant layer containing cholate, and virtually all of the cholate was removed from the pellet by the centrifugation step and subsequent dialy-

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Table 1

	Original mitochondria	DOL substituted mitochondria	DML substituted mitochondria
* Calculated substitution (%)	–	94	94
Observed substitution (%)	–	90	90
Lipid protein ratio (mg lipid/mg protein)	0.43	0.36	0.25
Protein recovery (%)	(100)	90	80
% cholate removed after substitution	–	>99.9	>99.9
BDH activity at 37°C (i.u./mg)	0.027	0.051	0.015

7.5 mg of mitochondria were incubated with 50 mg of lipid, 25 mg of potassium cholate pH 8.0 in a total volume of 1 ml sucrose buffer containing 2 mM ATP, 2 mM $MgSO_4$ and 0.1 mM dithiothreitol, at 4°C for 30 min. This incubation mixture was loaded onto a discontinuous sucrose density gradient of 0.1 ml of 80% sucrose and 1.0 ml of 20% sucrose, both solutions containing 2 mM ATP, 2 mM $MgSO_4$ and 0.1 M Tris–HCl buffer, pH 7.2. Centrifugation was carried out at 160 000 g_{av} for 20 hr at 4°C. The mitochondrial pellet with substituted lipid was collected at the boundary of the 20% and 80% sucrose layers.

* Calculated by assuming complete equilibration of the lipid pools.

sis. The lipid composition of the isolated pellet was close to that expected for complete equilibration of the exogenous and endogenous lipid pools. Experimental details are given in table 1.

The lipid-substituted complexes were then titrated with a large excess of the test lipid in the presence of cholate (0.5 mg/mg lipid) in sucrose buffer at 4°C, so that the test lipid comprised more than 96% of the equilibrated pool. Aliquots of the titration mixture were diluted 400-fold into the BDH assay medium. After this dilution less than 5% of the cholate in the aliquot remained bound to the titration complex, [see 5,6]. The enzyme remains membrane-bound in the assay medium; it sediments to form a pellet after 1 hr at 100 000 *g*.

As an alternative to using cholate for equilibration, a fusion technique was used to introduce DML into the mitochondrial membrane. DML (50 mg/ml) in sucrose buffer was sonicated at 20–25°C to optical clarity under nitrogen in a glass vial. This lipid suspension (200 μl) was added immediately to 50 μl of the mitochondria (50 mg/ml) and the mixture maintained at 37°C for 12 min: the final lipid composition was 96% DML and 4% endogenous lipid.

β-hydroxybutyrate dehydrogenase was assayed in a buffer containing 100 mM Tris–HCl, 0.1 M β-hydroxybutyrate, 1 mg/ml NAD^+ and 0.1 mM KCN. The pH was adjusted to 8.0 at all assay temperatures and the concentrations of both substrates were saturating under all conditions.

The protein, lipid and cholate determinations were performed as described previously [4]. DOL and DML were synthesised by the method of Robles and Van den Berg [7]; treatment of DOL with phospholipase D in the presence of 5% ethanolamine, followed by silicic acid chromatography yielded pure sample of dioleoyl phosphatidic acid and dioleoyl phosphatidyl ethanolamine. All other phospholipids were obtained from Lipid Products and cholesterol was obtained from Sigma.

3. Results

The lipids of ox heart mitochondria can be substituted with DOL or DML to levels which correspond to almost complete equilibration with the endogenous lipid. The specific activity of BDH at 37°C is 0.027 i.u/mg for the original mitochondria, compared with 0.051 i.u/mg and 0.015 i.u/mg for the DOL- and DML-substituted complexes. These activities are not affected by extensive dialysis against Amberlite XAD-2

Table 2
Lipids not supporting β-hydroxybutyrate dehydrogenase activity

Dioleoyl phosphatidic acid	Dioleoyl phosphatidyl ethanolamine	Cholesterol
Phosphatidyl serine	Phosphatidyl glycerol	Cardiolipin
Phosphatidyl inositol	Sphingomyelin	Cerebroside

The lipid composition of the titration complexes were 96% added lipid, 3.4% dioleoyl lecithin and 0.6% endogenous mitochondrial lipids. There was no observable BDH activity with any of these complexes (< 3% activity of BDH in DOL-mitochondria). Phospholipids of unspecified side chains are of bovine origin except for phosphatidyl glycerol which is derived from egg lecithin.

resin to remove residual cholate, nor by the addition of cholate to the complexes at 0.5 mg cholate/mg lipid. In this system cholate therefore acts as an inert mixing agent which merely accelerates the equilibration of the lipid pools.

Titrations to determine the lipid headgroup specificity for BDH activity showed that 96% of the lipid pool consisted of the added test lipid, activity was only maintained by lecithin (table 2). However, the activity of all the titration complexes in Table 2 could be restored to more than 90% of the original activity of the DOL-complex by back-titration with a large excess of DOL to 92% of the total lipid pool. The inhibitory actions of cholesterol and the phospholipids in Table 2 are therefore fully reversible and are not due to denaturation of BDH.

Similar titrations were performed to determine the effect of lecithin chain structure on BDH activity at 37°C (fig.1). The activities measured in the assay medium were unaffected by the use of cholate to equilibrate the lipid pools: washing the complexes to remove cholate before assay did not change the activity of BDH. Back titration with DOL to 92% of the total lipid restored the activity of all the lecithin titration complexes to at least 90% of the activity of the DOL-substituted mitochondria. At 37°C titration complexes with DOL or dilauroyl lecithin (DLL) activated BDH compared with the endogenous mitochondrial lipids. On the other hand DML and dipalmitoyl lecithin (DPL) lowered BDH activity and distearoyl lecithin (DSL) and dicaproyl lecithin (DCL) both completely inhibited the enzyme. These results suggested that BDH activity is relatively high only in complexes with lipids well above their transition temperatures (DOL and DLL), although the inactive complex with DCL suggests that the enzyme also requires a minimal lipid chain length to support activity. DSL, which has a transition temperature at 62°C, forms a completely inactive complex with BDH when assayed at 37°C suggesting that inhibition might occur when the lipid chains are in the crystalline state.

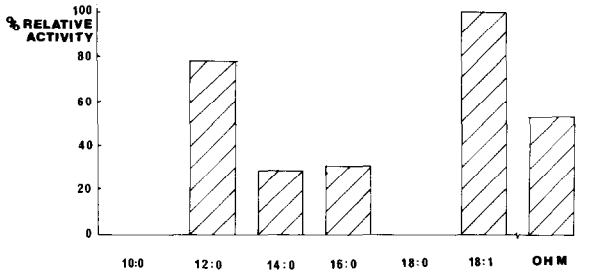


Fig.1. The effect of lecithin chain structure on β-hydroxybutyrate dehydrogenase activity. The activities of BDH at 37°C in native ox heart mitochondria (OHM) and in titration complexes with defined lecithins are given relative to DOL-substituted mitochondria (100% = 0.051 i.u./mg). Both side chains in each of the defined lecithins have the same structure and the titration complexes contained 96% added lecithin, 3.4% di-oleoyl lecithin and 0.6% endogenous mitochondrial lipids.

To examine this further, the temperature dependence of the BDH activity in lecithin complexes was examined. DOL and DLL complexes both show activity at all assay temperatures down to at least 5°C and were similar in this respect to BDH activity in the original mitochondrial fraction. The DML and DPL complexes were inactive below 24°C and 29°C respectively (fig.2), and the DSL complex only showed activity above 50°C. These temperature profiles were unaffected by extensively washing the complexes by centrifugation and resuspension, or dialysis, to remove residual cholate. The fusion experiment with

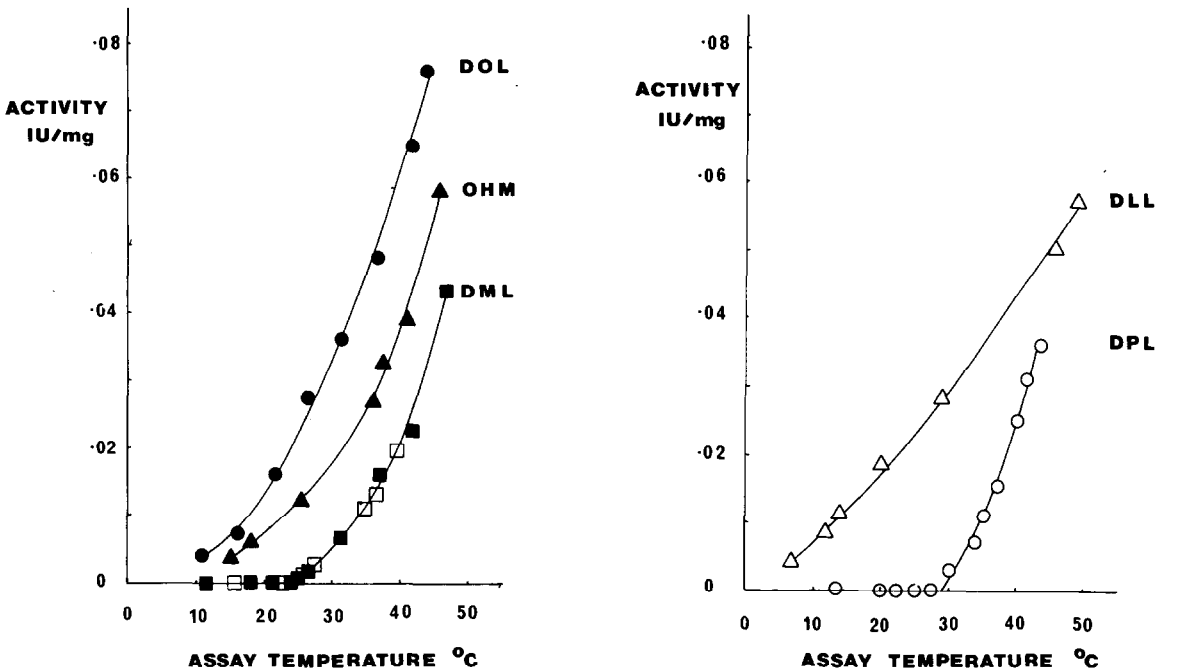
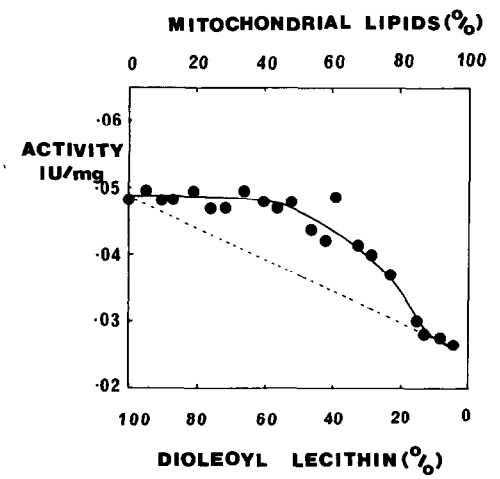


Fig.2. The temperature dependence of BDH activity in titration and fusion complexes of mitochondria with defined lipids. (a) (•) 99.6% DOL and 0.4% endogenous lipid; (■) 99.6% DML and 0.4% endogenous lipid; (□) fusion complex containing 96% and 4% endogenous lipid; (▲) native ox heart mitochondria. (b) (△) 96% DLL, 3.6% DOL and 0.4% endogenous lipid; (○) 96% DPL, 3.6% DOL and 0.4% endogenous lipid.

DML confirmed that the temperature profile for the DML titration complex was not attributable to the use of cholate: the BDH activity of the complex formed by fusion with DML was completely inhibited below 24°C (fig.2).



Experiments were also performed to determine whether BDH activity is a linear function of the lipid composition surrounding the protein. DOL-substituted mitochondria were titrated with mixtures of DOL and mitochondrial lipids in the presence of cholate, such that the total lipid, cholate and protein concentration were constant. The change in activity is markedly non-linear with respect to lipid composition, with the BDH activity remaining unaltered in the presence of an equimolar proportion of mitochondrial lipids (fig.3).

Fig.3. BDH activity in titration complexes of mitochondria with mixtures of DOL and mitochondrial lipids. 0.055 mg of DOL-mitochondria were incubated with 0.25 mg of cholate and 0.5 mg of lipid consisting of DOL and mitochondrial lipids (total extract with 2:1 chloroform/methanol), in the proportions shown in a total volume of 50 μl. The BDH activities were the same after 30 min, 1 hr and 24 hr incubations at 4°C in sucrose buffer. The dashed line defines a linear response in BDH activity to the lipid composition.

The activities were unaltered during 24 hr incubation period with cholate at 4°C at either 0.5 or 1.0 mg cholate/mg lipid. Similar results were obtained when DOL in cholate was added to native mitochondria. Although there are several possible explanations for these results, we think it is likely that BDH is able to select and/or retain a specific lipid environment after exposure to cholate concentrations which would cause complete mixing of the lipid pools in the absence of the protein.

4. Discussion

In the lipid substitution and titration techniques used to determine the effect of lipid structure on BDH activity, cholate acts as an ideal mixing agent which catalyses lipid exchange, without itself modulating enzymatic activity. On addition to mitochondria, cholate has virtually no effect on BDH activity at the concentrations used, but causes a rapid and complete equilibration of the endogenous mitochondrial lipids with the added lipids. Another general feature of the techniques used is that BDH activity is maintained throughout the lipid substitution procedures, and that the lipid binding sites on the protein remain inaccessible to the aqueous environment. In titration experiments with lipids which inactivated BDH, the activity could be fully recovered by back-titration with an excess of dioleoyl lecithin. We therefore, consider that the techniques used will yield complexes in which the structural interaction of lipid with BDH is very similar to that in the original membrane.

A significant feature of the interaction of BDH with some of the saturated lecithins is the complete inhibition of BDH activity below a temperature determined by the chain length. It seems very probable that the temperature at which inhibition occurs represents the crystallisation of the lipid molecules in the annulus interacting directly with the protein [5,8]. For DPL and DSL this temperature is at least 10–15°C below the lipid transition temperature in the absence of protein. We attribute this depression to the effect of the protein on the single shell of lipid bilayer which comprises the annulus [8]. BDH activity is higher than in the original membrane when complexed with lecithins substantially above their transition temperature (DOL and DLL), although the inactivity of the complex with DCL (10:0; 10:0 chains) probably implies that a minimum chain length is essential to maintain an active conformation similar to that in vivo.

These results provide an interesting contrast with those of Fleischer et al. [1], who found that BDH activity was maintained below the transition temperature of the saturated lecithins, and that the activity of several complexes showed biphasic temperature dependence below 50°C, substantially different from the monotonic temperature profiles shown here (figs.2a,b). We can rationalise these differences if we assume that the lipid substitution techniques we have used permit the normal interaction of BDH with both the choline headgroup *and* the lipid chains, so that the activity of the enzyme can be regulated by chain length and conformation. We interpret the data of Fleischer et al. [1] to imply that when the soluble apo-enzyme interacts with preformed lecithin bilayers, the interaction is predominantly through the choline headgroup, which we have confirmed here is necessary for activity. However, if the apo-enzyme does not interact with the lipid chains, either because of a conformation change, or its inability to penetrate a preformed bilayer, this would account for the insensitivity for BDH activity to the phase transition and the decrease in BDH activity above an optimal temperature, which we attribute to a dissociation of BDH from the choline headgroups, likely to be a predominantly ionic interaction. In addition, similar activation energies of 16–19 Kcal/mol were reported by Fleischer et al. [1] for all the complexes of the apoenzyme with different lecithins. Our value for the activation energy of BDH in the native mitochondria agrees closely with that of Fleischer et al., but we observe a wide range of values from 7–74 Kcal/mol for our titration complexes, depending on the structure of the lecithin chains.

These differences between our data and that of Fleischer et al. [1] point to one of the several unconsidered problems in attempts to determine lipid specificity by reactivation of an inactive enzyme. In general reactivation requires at least three steps:

(i) The protein must be presented with the lipid in a structural form with which it can interact.

(ii) The lipid must be able to bring about the change in protein conformation necessary to reactivate the enzyme: i.e. the structural interaction may be appropriate but the activation energy for the conformationed change required may still be very high.

(iii) The structural interaction of the lipid with the protein must be shown to be the same as in the native membrane.

We regard the lipid exchange techniques used here as a useful means of avoiding some of these problems, and we emphasise that the reactivation of inactive titration complexes is an essential requirement to demonstrate lipid specificity. In the experiments with BDH, the coincidence of the temperature profiles of BDH activity obtained either by titration or by fusion with DML is powerful supporting evidence that the complexes prepared by the use of detergent have similar structural interactions between the lipid and BDH as in the native membrane.

A final point of interest is the remarkable similarity between the temperature dependence of the BDH complexes with DPL and DML and the corresponding lipid complexes with the (Ca^{2+}, Mg^{2+}) ATPase from sarcoplasmic reticulum [5,8]. Both enzymes become inactive at 29°C in complexes with DPL and at 25°C when complexed with DML. We believe that many penetrant proteins may be sealed into the bilayer by using a similar structural interaction between the lipids in the annulus and the hydrophobic surface of the protein. It would not then be surprising to find a similar depression of the transition temperature of the lipids in the annulus for proteins with quite different specific functions. We will present further evidence for the structure and functional significance of the lipid annulus elsewhere.

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