Interactions between apo-(D- β -hydroxybutyrate dehydrogenase) and phospholipids studied by intrinsic and extrinsic fluorescence

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Interactions of D- β -hydroxybutyrate dehydrogenase with phospholipids were investigated by both intrinsic and extrinsic-fluorescence approaches. The intrinsic fluorescence, mainly caused by tryptophan residues, increased upon re-activation in the presence of phospholipids bearing a positive charge, i.e. phosphatidylcholine, but decreased in the presence of non-re-activating phospholipids with a negative charge. This indicates either that the environment of tryptophan residues is affected by charges rather than by hydrophobic chains of phospholipids, or that the enzyme undergoes different conformational changes depending on the nature of the phospholipids. On the other hand, the graph of the temperature-dependence of the fluorescence intensities of the enzyme embedded in dimyristoylphosphatidylcholine liposomes exhibits a break around 21 °C. This indicates either that at least one tryptophan residue is closely in contact with the hydrophobic chains of phospholipids or that there is a change in the environment of tryptophan residues owing to the physical state of the phospholipids. The addition of D- β -hydroxybutyrate apo-dehydrogenase to phospholipid liposomes containing diphenylhexatriene (a fluorescent probe) increased the diphenylhexatriene fluorescence polarization. Moreover, there was a partial fluorescence energy transfer from tryptophan to diphenylhexatriene. These results strongly favour the possibility that there is a portion of the enzyme polypeptide chain inserted into the phospholipid hydrophobic region. All these results demonstrate that D- β -hydroxybutyrate apo-dehydrogenase interacts with both polar and hydrophobic parts of phospholipids and leads to small, but essential, conformational changes of the enzyme.

INTRODUCTION

D- β -Hydroxybutyrate dehydrogenase from mitochondrial inner membrane [1,2] requires phosphatidylcholine or liposomes containing phosphatidylcholine for its activity [3]. It has been previously shown that the enzyme has a specific requirement for the choline head of phosphatidylcholine [4] and that the presence of other mitochondrial phospholipids, i.e. mainly phosphatidylethanolamine and cardiolipin, together or each mixed with phosphatidylcholine, increased both the activity and the efficiency of re-activation [3]. Moreover, the negatively charged phospholipids, such as cardiolipins, appear to be involved in the enzyme insertion into phospholipid monolayers [5]. This phospholipid requirement makes D- β -hydroxybutyrate dehydrogenase an interesting model for studying phospholipid–protein interactions. So far, D- β -hydroxybutyrate dehydrogenase–phospholipid interactions have been investigated by different approaches such as kinetic measurements of D- β -hydroxybutyrate dehydrogenase re-activation by phospholipids [4,6–8] or by detergents [9,10], ^{13}C n.m.r. [11], specific chemical modifications of essential amino acid residues [12,13], controlled proteolysis [14], apo-enzyme insertion into phospholipid monolayers [5] and interactions of the apo-dehydrogenase with photo-activable phospholipids [15].

Fluorescence is a very powerful technique used in the investigation of protein motions in a defined micro-environment and in the study of lipid–protein interactions

[16–19]. In order to obtain more information concerning the role of phospholipids in D- β -hydroxybutyrate dehydrogenase structure and in its insertion into liposomes, we report a fluorescence study of the interaction of the purified rat liver enzyme with phospholipids. Various fluorescence properties were investigated: fluorescence intensity, since D- β -hydroxybutyrate dehydrogenase contains four tryptophan residues [20], fluorescence polarization and fluorescence energy transfer, by using different fluorescent molecules such as tryptophan residues of the enzyme for intrinsic fluorescence and 1,6-diphenylhexa-1,3,5-triene as a hydrophobic fluorescent probe of the phospholipid bilayer.

Our results reveal the importance of membrane surface charges on the structural conformation of the enzyme and the interaction of a polypeptide domain with the hydrophobic part of the phospholipid bilayer.

MATERIALS AND METHODS

Materials

1,6-Diphenylhexa-1,3,5-triene was from Aldrich (Beerse, Belgium); EDTA and Tris were from Fluka AG, Buchs SG (Switzerland); 1,2-dimyristoylphosphatidylcholine, 1-oleoyl-2-stearoylphosphatidylcholine and phosphatidylserine were from Sigma (St. Louis, MO, U.S.A.); Hepes was from Boehringer (Mannheim, Germany). Other products were the highest grade quality available.

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Methods

D- β -Hydroxybutyrate dehydrogenase of rat liver mitochondria was purified close to homogeneity by the method of Bock & Fleischer [20], as slightly modified by Brenner *et al.* [21], and stored in liquid N₂ in 1 mM-EDTA/400 mM-LiBr/5 mM-dithiothreitol/5 mM-Hepes buffer, pH 7. Protein was measured by the method of Lowry *et al.* [22]. Natural phospholipids, i.e. phosphatidylcholine, phosphatidylethanolamine and cardiolipin, were prepared from freshly isolated rat liver mitochondria as described by Rouser & Fleischer [23]. Before use, phospholipids were micro-dispersed by sonication under a stream of N₂ in 1 mM-EDTA/20 mM-Tris buffer, pH 8.1 [24]. Phospholipid P was determined as described by Chen *et al.* [25].

Reconstituted D- β -hydroxybutyrate dehydrogenase-phospholipid complexes were prepared at 30 °C by incubation (30 min) of lipid-free apoenzyme with phospholipids in 1 mM-EDTA/5 mM-dithiothreitol/20 mM-LiBr/20 mM-Tris buffer, pH 8.1. Phospholipid complexes were prepared as follows: 2 mM-1,6-diphenylhexa-1,3,5-triene in tetrahydrofuran was micro-dispersed in 1 mM-EDTA/20 mM-Tris buffer, pH 8.1, with stirring to obtain a final concentration of 5 μ M-1,6-diphenylhexa-1,3,5-triene; a portion of this micro-dispersed sample was added to liposomes and the mixture was incubated for 30 min in the dark with stirring for incorporation of 1,6-diphenylhexa-1,3,5-triene into liposomes.

Fluorescence measurements were carried out with a mk. II FICA differential spectrofluorimeter with automatic correction of excitation. Samples were added in quartz cuvettes (5 mm $\times$ 5 mm). Fluorescence polarization of 1,6-diphenylhexa-1,3,5-triene was measured at 20 °C with an ELSINT MV I A fluorimeter with 342 nm and 430 nm filters for excitation and emission respectively. Fluorescence anisotropy (r) was calculated from the equation:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities detected with the analyser oriented parallel or perpendicularly to the direction of the polarized excitation beam. The microviscosity parameter $[(r_0/r) - 1]^{-1}$ was calculated by using $r_0 = 0.362$ as the maximum value of 1,6-diphenylhexa-1,3,5-triene anisotropy [26].

RESULTS

Dependence of D- β -hydroxybutyrate dehydrogenase intrinsic fluorescence on phospholipids

The emission of intrinsic fluorescence of D- β -hydroxybutyrate dehydrogenase (Fig. 1) had a maximum at 340 nm, which was related to the fluorescence of tryptophan residues. The reconstitution of D- β -hydroxybutyrate dehydrogenase-phospholipid complexes with mitochondrial phospholipids or phosphatidylcholine increased the fluorescence intensity, whereas phosphatidylethanolamine/diphosphatidylglycerol strongly decreased the fluorescence intensity. The effect of different natural or defined synthetic phospholipid on intrinsic fluorescence intensity of D- β -hydroxybutyrate dehydrogenase is quantified in Table 1. Zwitterionic phospholipids, i.e. mitochondrial phosphatidylcholine or synthetic

Fig. 1. Intrinsic-fluorescence emission spectra of D- β -hydroxybutyrate dehydrogenase (BDH); effect of phospholipid

Lipid-free enzyme (0.65 nmol) was incubated at 25 °C with a 100-fold molar excess of phospholipids in 200 μ l of 1 mM-EDTA/5 mM-dithiothreitol/50 mM-LiBr/20 mM-Tris buffer, pH 8.1. After 30 min incubation, samples were diluted to 500 μ l with the same medium and the fluorescence emission was measured at 25 °C (excitation 295 nm). In MPL, the PC/PE/DPG molar proportions were 4:4:1, and the PE/DPG molar ratio was 4:1 (for abbreviations see Table 1 legend).

unsaturated (1-oleoyl-2-stearoylphosphatidylcholine) and saturated (1,2-dimyristoylphosphatidylcholine) phosphatidylcholines increased the fluorescence intensity to about the same extent. In contrast, negatively charged phospholipid (phosphatidylethanolamine/

Table 1. Influence of different phospholipids on the intrinsic fluorescence intensity of D- β -hydroxybutyrate dehydrogenase

Apoenzyme (1.55 μ l) was incubated for 30 min at 30 °C with phospholipid in 200 μ l of buffer containing 20 mM-Tris/HCl, 1 mM-EDTA, 5 mM-dithiothreitol and 20 mM-LiBr, pH 8.1. After incubation, the samples were diluted to 500 μ l with the buffer. The fluorescence emission intensity measurements were done at 30 °C at 340 nm (295 nm as excitation wavelength); F_0 and F represent the fluorescence intensities in the absence and in the presence of phospholipid respectively. Abbreviations: PC, phosphatidylcholine; OSPC, 1-oleoyl-2-stearoylphosphatidylcholine; DMPC, 1,2-dimyristoylphosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PS, phosphatidylserine; MPL, mitochondrial phospholipids.

Experimental conditions	Phospholipid/enzyme molar ratio	F/F_0
Apoenzyme	—	1.00
+ mitochondrial PC	200	1.12
+ OSPC	200	1.10
+ DMPC	200	1.10
+ PE/DPG	190	0.66
+ PS	200	0.44
+ MPL	80	1.20

Fig. 2. Relative fluorescence intensity of D-β-hydroxybutyrate dehydrogenase in the presence of increasing amounts of phospholipids

For details, see the legend of Fig. 1.

diphosphatidylglycerol) and phosphatidylserine strongly decreased the fluorescence intensity. On the other hand, the D-β-hydroxybutyrate dehydrogenase–phospholipid complex reconstituted with mitochondrial phospholipids, which mimics the native enzyme, exhibited the highest fluorescence intensity. These variations in fluorescence intensity occurred without any shift of the maximum emission wavelength and reached a plateau at a high molar ratio of phospholipids/D-β-hydroxybutyrate dehydrogenase, as shown in Fig. 2. From these plots, the number of phospholipid molecules associated with the protein molecule was estimated to be about 80, 160 and 200 with mitochondrial phospholipids, phosphatidylcholine and phosphatidylethanolamine/diphosphatidylglycerol respectively.

The temperature-dependence of the fluorescence of the lipid-free D-β-hydroxybutyrate apo-dehydrogenase and the enzyme reconstituted with mitochondrial phospholipids, or with 1,2-dimyristoylphosphatidylcholine, was assessed in Fig. 3. When the temperature increased from 5 to 40 °C, the fluorescence intensity decreased linearly for both the lipid-free D-β-hydroxybutyrate apo-dehydrogenase and the mitochondrial-phospholipid–enzyme complex. In contrast, in the presence of 1,2-dimyristoylphosphatidylcholine, the graph of the fluorescence intensities exhibited a break around 21 °C corresponding to the transition temperature of 1,2-dimyristoylphosphatidylcholine bilayer [27].

Dependence of 1,6-diphenylhexa-1,3,5-triene fluorescence polarization on the addition of D-β-hydroxybutyrate dehydrogenase

The 1,6-diphenylhexa-1,3,5-triene fluorescence polarization (Table 2) was roughly the same with micro-dispersions of the different phospholipids tested in a fluid state (above their T_m), although these phospholipids were very different with regard to the structure of their polar head, i.e. mitochondrial phospholipids, phosphatidylcholine, 1-oleoyl-2-stearoylphosphatidylcholine, phos-

Fig. 3. Effect of temperature on intrinsic fluorescence of D-β-hydroxybutyrate dehydrogenase (BDH)

For details see the legend of Fig. 1. The BDH–phospholipid complexes were mixed at 30 °C and the fluorescence intensities were measured at the indicated temperature.

phatidylethanolamine/diphosphatidylglycerol or phosphatidylserine. The insertion of D-β-hydroxybutyrate dehydrogenase into phospholipid micro-dispersions significantly increased the polarization of 1,6-diphenylhexa-1,3,5-triene embedded in lipids. Furthermore (results not shown), the 1,6-diphenylhexa-1,3,5-triene fluorescence polarization increase was dependent on the increase in the D-β-hydroxybutyrate dehydrogenase/phospholipid molar ratio. Arrhenius plots of fluorescence polarization of 1,6-diphenylhexa-1,3,5-triene embedded

Table 2. Effect of insertion of D-β-hydroxybutyrate dehydrogenase on fluorescence polarization of 1,6-diphenylhexa-1,3,5-triene embedded into phospholipid vesicles

Lipid-free D-β-hydroxybutyrate dehydrogenase (9.5 μM) was incubated at 30 °C with 100 μM of phospholipids in 500 μl of 1 mM-EDTA/5 mM-dithiothreitol/20 mM-LiBr/20 mM-Tris buffer, pH 8.1. After 30 min incubation, fluorescence anisotropy (r) was measured at 20 °C, with excitation at 342 nm and emission at 430 nm. The molar ratio of phospholipids to 1,6-diphenylhexa-1,3,5-triene was 500:1. For abbreviations, see Table 1 legend.

Phospholipids	Apoenzyme	r	Δr
MPL	–	0.109	0.069
	+	0.178	
PC	–	0.099	0.034
	+	0.133	
PE/DPG	–	0.126	0.056
	+	0.182	
PS	–	0.140	0.085
	+	0.225	
OSPC	–	0.140	0.028
	+	0.168

Fig. 4. Effect of D- β -hydroxybutyrate dehydrogenase (BDH) on transition temperature of OSPC

For details and abbreviations, see the legend of Table 1.

in 1-oleoyl-2-stearoylphosphatidylcholine in the absence or in the presence of D- β -hydroxybutyrate dehydrogenase are shown in Fig. 4. The enzyme does not significantly modify the transition temperature of this phospholipid ($T_m = 8^\circ\text{C}$).

Energy transfer of tryptophan fluorescence to 1,6-diphenylhexa-1,3,5-triene

The topological relationships between domains of the D- β -hydroxybutyrate dehydrogenase polypeptide chain and the hydrophobic core of the phospholipid bilayer have been studied by fluorescence energy transfer from tryptophan to 1,6-diphenylhexa-1,3,5-triene. The effect of increasing concentration of 1,6-diphenylhexa-1,3,5-triene on D- β -hydroxybutyrate dehydrogenase intrinsic fluorescence in reconstituted D- β -hydroxybutyrate dehydrogenase-mitochondrial-phospholipid complexes is illustrated in Fig. 5 (curve A). The enzyme fluorescence quenching was related to an increase of 1,6-diphenylhexa-1,3,5-triene fluorescence on excitation at 295 nm (Fig. 5, curve B). This increase of fluorescence intensity is specifically due to an energy transfer from the fluorescence emission of tryptophan residues to the probe. Indeed, when the probe, embedded in mitochondrial-phospholipid liposomes, is directly excited at its excitation wavelength (360 nm), the fluorescence emission (intensity and maximum wavelength) is not significantly modified after subsequent insertion of the enzyme (results not shown).

Effect of NADH on intrinsic fluorescence of D- β -hydroxybutyrate dehydrogenase

D- β -Hydroxybutyrate dehydrogenase requires phosphatidylcholine for its catalytic activity [3], and the

Fig. 5. Fluorescence energy transfer from D- β -hydroxybutyrate dehydrogenase to 1,6-diphenylhexa-1,3,5-triene

Reconstituted enzyme-mitochondrial phospholipid complex (0.81 nmol of protein; 40 mol of phospholipid/mol of enzyme) was incubated at 25°C in 500 μl of 1 mM-EDTA/5 mM-dithiothreitol/20 mM-LiBr/20 mM-Tris buffer, pH 8.1, with different concentrations of 1,6-diphenylhexa-1,3,5-triene. After 30 min of incubation with stirring, fluorescence emissions at 340 nm (enzyme) and at 455 nm (1,6-diphenylhexa-1,3,5-triene; were measured at 25°C (excitation 295 nm). The results take account of the emission of 1,6-diphenylhexa-1,3,5-triene alone embedded in liposomes and excited at 295 nm. Curve A, quenching of enzyme intrinsic fluorescence by 1,6-diphenylhexa-1,3,5-triene; curve B, effect of enzyme on 1,6-diphenylhexa-1,3,5-triene fluorescence.

interaction of the enzyme with the phospholipid polar head leads to NAD(H) binding to the active site, which is a prerequisite for the catalysis [28]. The intrinsic fluorescence of either lipid-free D- β -hydroxybutyrate dehydrogenase or reconstituted D- β -hydroxybutyrate dehydrogenase-mitochondrial-phospholipid complex was strongly quenched by increasing NADH concentration, suggesting that the lipid-free enzyme could bind NADH as does the phospholipid-enzyme complex (Fig. 6).

DISCUSSION

Studies of protein/phospholipid interactions are a prerequisite for understanding the structure-function relationships of biological membranes. D- β -Hydroxybutyrate dehydrogenase-phospholipid interactions were investigated by using intrinsic and extrinsic fluorescence in order to study the effect of phospholipids on the enzyme structure and on its status when the enzyme is associated with lipids.

The intrinsic fluorescence of D- β -hydroxybutyrate dehydrogenase was affected by phospholipids. This change can be the consequence of a modification in the environment of tryptophan residue(s). The increase of intrinsic fluorescence intensity in the presence of

Fig. 6. Effect of NADH on D- β -hydroxybutyrate dehydrogenase (BDH) intrinsic fluorescence

Lipid-free enzyme or reconstituted enzyme–mitochondrial phospholipid complex (1.94 μ M; 80 mol of phospholipid/mol of enzyme) was incubated at 25 °C with the indicated concentrations of NADH in 500 μ l of 1 mM-EDTA/5 mM-dithiothreitol/20 mM-LiBr/20 mM-Tris buffer, pH 8.1. After 5 min of incubation, fluorescence emission was measured at 340 nm (excitation 295 nm). In the same conditions, controls were done with NADH alone and mixed with mitochondrial phospholipids (MPL).

phosphatidylcholine or a phospholipid containing phosphatidylcholine can be attributed to the zwitterionic surface charge potential, whereas the strong decrease in fluorescence intensity, observed in the presence of phosphatidylethanolamine/diphosphatidylglycerol or phosphatidylserine microdispersions, can be attributed to the highly negatively charged surface environment. Thus these variations in the surface charges according to the nature of phospholipid polar head composition can induce a conformational change in the D- β -hydroxybutyrate dehydrogenase polypeptide chain. In agreement with these results, we have shown that hydrophilic interactions between phospholipid polar heads and D- β -hydroxybutyrate dehydrogenase are essential for the penetration of the apoenzyme into phospholipid monolayers [5]. The highest D- β -hydroxybutyrate dehydrogenase fluorescence intensity increase obtained in the presence of mitochondrial phospholipid confirms the crucial role of phosphatidylcholine in the correct insertion of this enzyme, its conformation and, consequently, its biological function. The effect of phospholipids on the fluorescence intensity of tryptophan residues observed without shift of maximum emission wavelength has been previously obtained with cytochrome b_5 [16] and with apolipoprotein GL N₁ [29]. By using the fluorescence life-time technique (results not shown), we have observed that the fluorescence life-time of tryptophan residues of the enzyme increases significantly after insertion of the enzyme into phosphatidylcholine vesicles, whereas it remains unchanged in the enzyme reconstituted

with phosphatidylethanolamine/diphosphatidylglycerol micro-dispersions [30]. These results are in agreement with the above conclusions.

The fluorescence polarization increase of 1,6-diphenylhexa-1,3,5-triene embedded within liposomes on addition of D- β -hydroxybutyrate dehydrogenase which means that the motion for the probe becomes more constrained, is explained as a consequence of the proximity of 1,6-diphenylhexa-1,3,5-triene to enzyme molecules and is a strong argument for a penetration of a part of the enzyme polypeptide chain into the phospholipid hydrophobic core. Similar results have been obtained with bovine heart D- β -hydroxybutyrate dehydrogenase [7], but the polarization extent appears higher with rat liver D- β -hydroxybutyrate dehydrogenase (the present work). This could indicate a significant difference of liposome penetration between the rat liver enzyme and the bovine heart enzyme. In agreement with results obtained with the bovine heart D- β -hydroxybutyrate dehydrogenase [7], there is no significant shift of transition temperature of synthetic phospholipid induced by the insertion of the apoenzyme into such phospholipid vesicles.

The study of fluorescence energy transfer has often been used to investigate protein–lipid interactions [31–34]. In this present work, the experiments have been carried out with the reconstituted D- β -hydroxybutyrate dehydrogenase–mitochondrial-phospholipids–1,6-diphenylhexa-1,3,5-triene complex, in order to estimate the extent of penetration of the enzyme into liposomes. The results demonstrate an efficient fluorescence energy transfer from tryptophan residues to 1,6-diphenylhexa-1,3,5-triene. Since the 1,6-diphenylhexa-1,3,5-triene molecules are buried in the hydrophobic region of the phospholipid bilayer [35], the results suggest the proximity of some tryptophan residues of the protein to the hydrophobic part of phospholipids. On the other hand, to our present knowledge, one cannot exclude an energy transfer from a tryptophan residue, located in the polar group region, close enough to 1,6-diphenylhexa-1,3,5-triene. The answer would need further study in order to estimate the Forster distance (R_0) and the energy efficiency (E) between the donor and the acceptor [36]. These data are not easy to obtain. In addition, changes observed in the fluorescence intensities of tryptophan residues related to phospholipid fluidity state strongly support that the enzyme inserts into the lipid hydrocarbon chain region, although a change in the environment of tryptophan residues could not be excluded. Dufourcq *et al.* [16] have concluded that the sensitivity of cytochrome b_5 to the phase transition is a consequence of the protein penetration in the hydrophobic core of the bilayer. In agreement with these conclusions, we previously demonstrated [15], using photolabelling of D- β -hydroxybutyrate dehydrogenase with synthetic azidoaryl-phospholipids, that a portion of the enzyme polypeptide chain directly interacts with the hydrophobic moiety of phospholipids.

The quenching of D- β -hydroxybutyrate dehydrogenase intrinsic fluorescence intensity, owing to the addition of NADH to both lipid-free enzyme or reconstituted enzyme–mitochondrial-phospholipid complex, shows that NADH can bind to the enzyme even in the absence of phospholipids. This result contrasts with those obtained several years ago with bovine heart enzyme [28]. Our results obtained with the rat liver enzyme can be explained by the fact that NADH is able to bind to the lipid-free enzyme, but does not permit the oxidoreduction

process in the absence of phospholipids. In a study of the bovine heart enzyme, Fritzsche *et al.* [37], using e.s.r. and fluorescence techniques, have indicated that phosphatidylcholine could simply modify the reorientation of the coenzyme-binding site to the correct state for catalytic activity.

In conclusion, this fluorescence study of the structure-function relationships of D- β -hydroxybutyrate dehydrogenase with phospholipids revealed that:

(1) the enzyme undergoes a conformational change, which depends on the nature of the phospholipid charge;

(2) part of the enzyme polypeptide chain is buried in the phospholipid hydrophobic core;

(3) although inactive, the lipid-free enzyme is able to bind NAD(H) as does the active enzyme-mitochondrial-phospholipid complex.

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