# Photolabelling of D-β-hydroxybutyrate apodehydrogenase with azidoaryl phospholipids

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Two synthetic photoactive azidoarylphosphatidylcholines were used to investigate the level of interaction between D-β-hydroxybutyrate apodehydrogenase (apoBDH), an amphipathic membrane protein, with the hydrophobic domain of phospholipids. The two synthetic lecithins, PL I (1-myristoyl-2-12-*N*-(4-azido-2-nitrophenyl)aminododecanoyl-*sn*-glycero-3-phosphocholine) and PL II (1-myristoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-phosphocholine), are able to reactivate the non-active purified apoBDH as well as the non-photoactive homologs, indicating that the photoreactive chemical groups are without effect on the cofactor properties of phosphatidylcholine. Photoirradiation of reconstituted complexes between phospholipid containing azidoaryllecithin and apoBDH leads to a covalent binding of some synthetic lecithin molecules on the protein. The labelling, about 3 times higher with PL II than with PL I, suggests that the area of interacting domain of BDH with the hydrophobic moiety of phospholipid is more important at or near the surface of the lipid bilayer than in the inner part. This approach is further demonstration that BDH is an integral protein.

*D-β-Hydroxybutyrate apodehydrogenase* *Photolabelling* *Azidoaryl phospholipid*

## 1. INTRODUCTION

BDH from mitochondria is a membrane-bound enzyme requiring lecithin for its activity [1]. BDH-phospholipid interactions have been actively studied for a long time using several methodological approaches like kinetics of reactivation [2–5], chemical modification of essential amino acid residues [6–8], proteolysis [9], NMR of carbon-13 labelled in phospholipids [10], intrinsic and extrinsic fluorescence [11,12] and penetration of BDH into phospholipid monolayers [13]. Taking into account the fact that the phospholipid fatty acid moiety does not present any chemical reactivity, it appears difficult to study directly the interaction of the hydrophobic domain of membrane protein like BDH with phospholipids. Thus, we synthesized two photoactive azidoarylphosphatidylcholines containing the photoactive chemical group at different levels in the hydrophobic part.

Here, we used these valuable tools in order to investigate the degree of penetration of BDH into the phospholipid bilayer.

Previously, azidoarylphospholipids were successfully used in studies of interactions of phospholipids with several membrane proteins like cytochrome $b_5$ [14], cytochrome *c* oxidase [15], succinate dehydrogenase [16], ATPase complexes [17,

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*Abbreviations:* ApoBDH, D-β-hydroxybutyrate apodehydrogenase; BDH, D-β-hydroxybutyrate dehydrogenase; DMPC, dimyristoylphosphatidylcholine; DPG, diphosphatidylglycerol; lyso-DMPC, lysomyristoylphosphatidylcholine; MPL, mitochondrial phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL I, 1-myristoyl-2-12-*N*-(4-azido-2-nitrophenyl)aminododecanoyl-*sn*-glycero-3-phosphocholine; PL II, 1-myristoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-phosphocholine



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18], apolipoproteins [19] and phosphatidylcholine exchange protein [20].

## 2. MATERIALS AND METHODS

Lipid-free hydrosoluble apoBDH was purified from rat liver mitochondria close to homogeneity according to the method of Bock and Fleischer [21] slightly modified by Brenner et al. [22]. Proteins were estimated according to the method of Lowry et al. [23] modified by Ross and Schatz [24] for the samples with dithiothreitol. Mitochondrial phospholipids were purified according to Rouser and Fleischer [25] from freshly prepared mitochondria. Non-radioactive azidoarylphospholipids (PL I and PL II) were synthesized and controlled as described by Bisson and Montecucco [26]. We found here that lipid phosphorus determination by the sensitive micromethod of Chen et al. [27] was quite sensitive enough to estimate phospholipids, so we did not use radioactive phospholipids. However, complete delipidation of the non-covalently bound phospholipid molecules of BDH-phospholipid complexes was carefully checked after irradiation by previous addition of [$^{14}$C]lecithin in the phospholipid preparation as internal marker. Phospholipid liposomes containing PL I and PL II in presence of [$^{14}$C]PC and mitochondrial phospholipids (for exact composition see legend of tables 1,2) were prepared in 20 mM Tris, 1 mM EDTA (pH 8.1) by sonication under nitrogen stream as described in [28]. Reconstituted BDH-phospholipid or cytochrome *c*-phospholipid complexes were obtained by incubating lipid-free enzyme in the dark with phospholipids containing PL I or PL II for 10 min at 30°C in a medium containing 20 mM Tris, 1 mM EDTA, 5 mM dithiothreitol (pH 8.1); the samples were rotated nearly horizontally in order to make a continuously changed film on the tube wall and then irradiated for 20 min at 0°C with a 250 W Osram halogen lamp through a glass/water filter in order to eliminate radiation lower than 300 nm. After irradiation an aliquot was taken for enzymatic activity measurement and the rest was precipitated with 7% $HClO_4$ at 0°C for 10 min and centrifuged at 7500 rpm for 10 min in a clinical centrifuge; the pellet was washed with absolute ethanol at 0°C and centrifuged 10 min; the washing was repeated 3 times in order to remove all the phospholipids non-covalently bound to BDH; the final pellet was dissolved in 0.1 N NaOH and aliquots were used for protein and phospholipids estimation and for radioactivity measurement. The yield of phospholipids extraction was estimated $\geqslant$99% by determination of the radioactivity remaining in the samples after precipitation and washing. The yield of protein was about 56%. Control samples were treated in parallel to the assay samples: one which did not contain azidoarylphospholipids (control 1) and another which contained neither protein nor azidoarylphospholipid (control 2). Control 1 was made in order to subtract traces of phosphorus which could be due to the non-covalently bound phospholipids; control 2 was done in order to correct the non-specific estimation of proteins. The enzymatic activities after and before irradiation were measured at 37°C as previously described [8]. The molar ratio of PL I or PL II bound to BDH or cytochrome *c* was calculated with an $M_r$ of 31000 for BDH [22] and 12400 for cytochrome *c*.

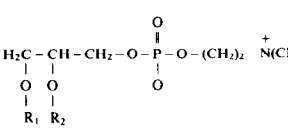
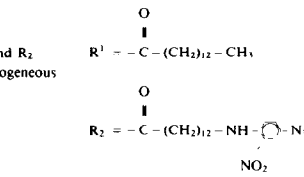
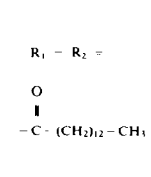
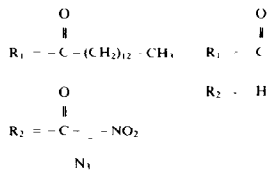
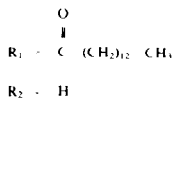
All chemical reagents used for azidoarylphospholipids synthesis were obtained as described by Bisson and Montecucco [26]. DMPC and lysoDMPC were from Sigma. Di[1-$^{14}$C]palmitoyl-L-$\alpha$-phosphatidylcholine (115 mCi/mmol) was from Amersham.

## 3. RESULTS AND DISCUSSION

The ability to reactivate purified apoBDH was compared, using photoactive phospholipids and their homologs (non-photoactive): DMPC for PL I and lysoDMPC for PL II. Table 1 indicates that PL I reactivated BDH as well as DMPC while PL II gave a higher BDH reactivation than lysoDMPC. This last result can be explained by the presence of the photoactive group in PL II. Although the extent of reactivation was only partial compared to the reactivation obtained with total mitochondrial phospholipids, as previously shown for DMPC and lysoDMPC [3], it can be concluded that the presence of an additive photochemical group in the hydrophobic part of lecithin does not prevent the reactivation process. In addition, the photoirradiation led to 20% decrease of its activity (table 2). Moreover, this inactivation is not due to the covalent binding of PL I or PL II to BDH since the same inactivation was obtained by photoirradiating the reconstituted BDH-MPL

Table 1

Comparison of reactivation ability of phospholipid containing lecithin or azidoaryl analogs on D-β-hydroxybutyrate apodehydrogenase

| Phospholipid structure | MPL[a] | PL I[b] | DMPC[c] | PL II[d] | LysoDMPC[e] |
|---|---|---|---|---|---|
|  | $R_1$ and $R_2$ heterogeneous |  |  |  |  |
| Relative enzymatic activity (%) | 100 | 52 | 51 | 35 | 11 |

ApoBDH (0.026 mg/ml final concentration) was preincubated in the dark at 30°C for 20 min in 250 μl of 20 mM Tris, 1 mM EDTA, 5 mM dithiothreitol (pH 8.1) containing the different phospholipids in the ratio of 60 μg P/mg protein. The compositions of the different phospholipid mixtures were as follows: [a] the total mitochondrial phospholipid (MPL) in the ratio of PC/PE/DPG/PI (0.4/0.35/0.20/0.05); [b] PL I mixed with the non reactivating mitochondrial phospholipid PLI/PE/DPG/PI (0.4/0.35/0.2/0.05); [c] DMPC mixed as in [b] DMPC/PE/DPG/PI (0.4/0.35/0.2/0.05). In assays [d] (pure PL II) and [e] (pure lysoDMPC) enzymatic activities were measured without preincubation of the enzyme with phospholipids. PL II and lysoDMPC were used at 30 μM (final concentration) below their CMC (about 120 μM as determined as described in [33]). At the end of the incubation an aliquot of 100 μl (2.6 μg protein) was taken for enzymatic activity measurement as described in section 2

Table 2

Photolabelling of D-β-hydroxybutyrate dehydrogenase or cytochrome *c* with azidoaryl phospholipids

| Membrane proteins | PL I | | PL II | |
|---|---|---|---|---|
| | Remaining activity after illumination[a] (%) | Phospholipid covalently bound[b] (molar ratio) | Remaining activity after illumination[a] (%) | Phospholipid covalently bound[b] (molar ratio) |
| D-β-Hydroxybutyrate apodehydrogenase | 77 | 6.47 ± 0.4 (3) | 82 | 18.6 ± 5.8 (3) |
| Cytochrome *c* | – | 2.27 ± 0.2 (2) | – | 1.72 ± 0.9 (3) |

[a] The remaining activity after irradiation was expressed in % compared to the activity before irradiation

[b] Values are given with the standard error. Number of experiments in parentheses

530 μg of lipid-free BDH or cytochrome *c* were incubated for 10 min at 30°C in 1.5 ml of 20 mM Tris, 1 mM EDTA, 5 mM dithiothreitol (pH 8.1) with sonicated phospholipid liposomes containing PL I or PL II in the following ratios: PL I/PC/PE/DPG/PI (0.1/0.3/0.35/0.2/0.05) or PL II/PC/PE/DPG/PI (0.02/0.38/0.35/0.2/0.05) in presence of traces of radioactive phosphatidylcholine as internal marker ($1.40 \times 10^6$ cpm). The amount of phospholipid in the samples was 25 μg lipid phosphorus/mg protein

complex without photoactivable probes (not shown).

The amounts of azidoarylphospholipids bound to the polypeptide chain of BDH are given in table 2. BDH binds to both photolabelled phospholipids. The binding of PL II with the reactive nitrene near the polar head-group was about 3 times higher than the binding of PL I where the nitrene

group is on the methyl terminus of one of the fatty acid chains.

A possible binding of these probes with proteins located outside the liposomes [29] was ruled out since the binding of PL I or PL II to cytochrome *c*, an associated protein, is much lower and does not significantly change with the position of the reactive nitrene group (table 2). Moreover, Bisson et al. [14] have reported that cytochrome $b_5$ is labelled only in its hydrophobic part; the same authors [15] have reported that the two extrinsic subunits of bovine heart cytochrome *c* oxidase do not bind PL I and PL II. Montecucco et al. [17] have found that neither PL I nor PL II labelled the F1 portion of mitochondrial ATPase complex.

Our results suggest that the interacting domain of BDH is less important with PL I than with PL II; this is in agreement with the fact that apoBDH is an amphipathic hydrosoluble protein [21] with a large part of the polypeptide chain outside the membrane while the rest of the polypeptide chain interacts with the phospholipid bilayer; it can be suggested that this interaction is due to a hydrophobic tail embedded in the bilayer, as previously shown for cytochrome $c_1$ [30], cytochrome $b_5$ [31] and acetylcholinesterase [32].

This is the first report for the direct interaction of BDH with hydrophobic part of phospholipids. Identification of portions of the BDH polypeptide chain interacting with PL I or PL II is under investigation by using radiolabelled photoreactive arylazidophospholipid and proteolysis experiments.

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