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THE ORIENTATION OF D-β-HYDROXYBUTYRATE DEHYDROGENASE IN THE MITOCHONDRIAL INNER MEMBRANE

J. OLIVER McINTYRE, HANS-GEORG O. BOCK and SIDNEY FLEISCHER

Department of Molecular Biology, Vanderbilt University, Nashville, Tenn. 37235 (U.S.A.)

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Summary

D-β-Hydroxybutyrate dehydrogenase of beef heart mitochondria is a lipid-requiring enzyme, bound to the inner membrane. The orientation of this enzyme in the membrane has been studied by comparing the characteristics of the enzyme in mitochondria and 'inside-out' submitochondrial vesicles. We observe that the enzymic activity is (1) latent in intact mitochondria; (2) relatively stable to trypsin digestion in mitochondria but rapidly inactivated in submitochondrial vesicles by this treatment; and (3) released more rapidly from submitochondrial vesicles by phospholipase A_2 digestion than from mitochondria. Conclusive evidence that D-β-hydroxybutyrate dehydrogenase is localized on the matrix face of the mitochondrial inner membrane is provided by the correlation that the enzyme is released from submitochondrial vesicles before the membrane becomes leaky to cytochrome *c*. The arrangement of D-β-hydroxybutyrate dehydrogenase in the membrane is discussed within a generalized classification of the orientation of proteins in membranes. The evidence indicates that D-β-hydroxybutyrate dehydrogenase is an amphipathic molecule and as such is inlaid in the membrane, i.e. the enzyme is partially inserted into the hydrophobic milieu of the membrane, with the polar, functional end extending into the aqueous milieu.

Introduction

Biological membranes are highly organized structures which consist mainly of proteins and phospholipids. Both the structure and function of biomembranes depend on the nature of the specific interactions between the protein

Abbreviations: PIPES, piperazine-*N*,*N*'-bis-(2-ethanesulfonic acid); HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid.

and phospholipid components. The lipids are generally considered to be organized as a bilayer with which the proteins are associated to a greater or lesser extent [1]. Fleischer and co-workers [2,3] have classified the protein components into two groups: (1) intrinsic membrane proteins which are components of the membrane superstructure and penetrate into or across the hydrophobic lipid bilayer; and (2) membrane-associated proteins which are not required for the structural integrity of the membrane and can be released without fully disrupting the membrane superstructure. The importance of the asymmetry of biological membranes to their function has become apparent [4,5]. The mitochondrial inner membrane, in particular, exhibits a distinct functional asymmetry in that a transmembrane proton-gradient can be generated by electron transport or ATP hydrolysis [5,6]. D-β-Hydroxybutyrate dehydrogenase is present in most mammalian tissues and has been found to be associated with the mitochondrial inner membrane [7]. It has been purified to homogenity from bovine heart mitochondria [8] and is one of the most intensively studied lipid-requiring enzyme [9,10]. This study is concerned with the nature of orientation of the enzyme in the mitochondrial inner membrane. The studies described include a novel procedure for characterizing the sidedness of D-β-hydroxybutyrate dehydrogenase in the membrane. A brief report of a portion of this work has appeared [11].

Materials and Methods

Sucrose, special enzyme grade, was obtained from Schwartz/Mann Co. (Orangeburg, N.Y.). DL-β-Hydroxybutyric acid (sodium salt), bovine serum albumin (Fraction V, powder), dithiothreitol, antimycin A, equine cytochrome *c* (Type III), Tris, glycylglycine, and PIPES (1,4-piperazine-diethanesulfonic acid) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Bovine serum albumin solution, from Armour Pharmaceutical Co. (Chicago, Ill.) was used as the protein standard. NAD^+ and NADH were obtained from P-L Biochemicals Inc, (Milwaukee, Wisc.). Trypsin (Grade B, essentially free of chymotrypsin) and HEPES (4-2-hydroxyethyl-1-piperazine-ethanesulfonic acid) were obtained from Calbiochem (San Diego, Calif.). *Naja naja siamensis* venom was obtained from Miami Serpentarium Laboratories (Miami, Fla.). LiBr and deoxycholic acid were obtained from Matheson, Coleman and Bell (Norwood, Ohio). The LiBr was prepared as a 4 M stock solution which was purified by filtration through an activated-carbon column and a 0.22 μm filter (Millipore Corporation, Bedford, Mass.). The deoxycholic acid was purified by recrystallization as previously described [12]. All other chemicals were reagent grade. All solutions were prepared in deionized water.

Assays. Protein was measured by the procedure of Lowry et al. [13] with bovine serum albumin as protein standard. When dithiothreitol, was present in the sample, the assay was carried out as described by Ross and Schatz [14] using iodoacetate to carboxymethylate the dithiothreitol, which would otherwise interfere with the assay for protein. Phosphorus was measured using a modification [15] of Chen et al. [16].

The D-β-hydroxybutyrate dehydrogenase activity of bovine heart mitochondria or submitochondrial vesicles was measured spectrophotometrically as

the rate of reduction of NAD^+ with D-β-hydroxybutyrate as substrate in hypotonic medium at 340 nm [17]. Approx. 100 μg of protein was added to a 1 ml cuvette (10 mm light path), preincubated at 37°C and containing, in 0.9 ml, a final concentration of 10 mM potassium phosphate (pH 7.35), 0.5 mM EDTA, 0.4 mg/ml bovine serum albumin, 1.27% (v/v) ethanol, 0.3 mM dithiothreitol, and 2 mM NAD^+. The sample was incubated in the cuvette for 3 min at 37°C with antimycin A (to block reoxidation of NADH by electron transport). The enzymic reaction was started by the addition of 0.1 ml of DL-β-hydroxybutyrate to a final concentration of 20 mM. The D-β-hydroxybutyrate dehydrogenase activity was measured either with or without preincubation of the sample for 15 min with mitochondrial phopholipid, as specified.

Succinate-cytochrome *c* reductase activity was measured at 32°C as described previously [18] except that the solution was made 0.25 M in sucrose to minimize rupture by osmotic shock. Succinate oxidase activity was measured at 32°C using a Clark oxygen electrode as described previously [19] except that 12 mM sodium succinate was used as substrate. Malate dehydrogenase and glutamate dehydrogenase were measured at 37°C using a method modified from Parsons and Williams [20]. Malate dehydrogenase activity cannot accurately by measured in intact mitochondria since NADH is not readily permeable through the membrane to the matrix space where this enzyme is localized [7]. Mitochondria were therefore solubilized in detergent by diluting, in a total volume of 0.1 ml, an aliquot of each sample to be assayed, to a final concentration of 0.5 mg protein and 0.25 mg deoxycholic acid per ml and 25 mM glycylglycine (pH 7.4). The membrane suspension 'clarified' and 0.5 μg of antimycin A was added to block oxidation of NADH by electron transport. The malate dehydrogenase activity was measured at 37°C in 5 mM oxaloacetate, 0.4 mM NADH and 50 mM potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of 10 μl (5 μg protein) of the detergent-solubilized mitochondria and the oxidation of NADH was measured at 340 nm. The supernatant fraction, following phospholipase A_2 digestion of mitochondria, was assayed for malate dehydrogenase activity without the addition of deoxycholic acid. Glutamate dehydrogenase was measured in a similar manner to malate dehydrogenase but in 5 mM α-ketoglutarate, 5 mM NH_4Cl, 0.1 mM NADH and 50 mM potassium phosphate buffer (pH 7.5).

Preparation of mitochondria and submitochondrial vesicles. Heavy bovine heart mitochondria were prepared on a large scale by the method of Blair [21] with modifications described previously [8] and were stored frozen at −80°C. Submitochondrial vesicles were prepared from these mitochondria by nitrogen compression-decompression and shear, using the Parr Bomb, as described by Fleischer et al. [22]. This procedure yields membrane vesicles oriented inside-out, that is, with the matrix side (inner face) of the mitochondrial inner membrane exposed to the aqueous medium. The mitochondria, used in the trypsin or phospholipase A_2 digestion studies reported here, were prepared fresh on a small scale (three beef hearts) for each experiment and were not frozen before use. However, similar results were obtained with mitochondria prepared on a large scale, frozen once, and stored at −80°C before being digested; repeated freezing and thawing increased the trypsin sensitivity of D-β-hydroxybutyrate dehydrogenase and the rate of release of this enzyme with phospholipase A_2.

Trypsin digestion of membranes. Trypsin was dissolved to 1 mg/ml in 1 mM $CaCl_2$, 10 mM KCl and the pH was adjusted to 3.0 with HCl. This solution was kept at room temperature for 3 h to inactivate any residual chymotrypsin and was then stored at 4° C overnight before use. Mitochondria or submitochondrial vesicles were suspended to 2 mg/ml in 0.25 M sucrose/20 mM Tris-HCl (pH 8.1)/5 mM dithiothreitol, and were preincubated for 10 min at room temperature. The digestion was initiated by the addition of an aliquot of the trypsin solution to a final concentration of 0.1 mg/ml (5% (w/w) on a protein basis). Aliquots were removed after varying periods of time and immediately assayed for D-β-hydroxybutyrate dehydrogenase activity. The sample was added directly to a cuvette (approximately a 20-fold dilution), preincubated at 37° C, and the enzymic reaction was initiated by the addition of substrate after 2 min incubation of the membrane with 0.5 μg of antimycin A (added to block electron transport). There was no significant difference in the time course of D-β-hydroxybutyrate dehydrogenase inactivation when trypsin digestion was blocked by the addition of soybean trypsin inhibitor (a 5-fold excess (w/w) trypsin) to the sample before it was assayed for enzyme activity.

The rate of proteolysis with trypsin was monitored with a Radiometer, type TTT2, pH-stat autotitrator. Digestions were carried out as described above but in the absence of buffer. The rate of production of protons in 2.0 ml of digestion mixture was monitored by the rate of addition of a dilute solution of NaOH (approx. 10 mM) which was required to maintain the pH at 8.1.

Phospholipase A_2 digestion of membranes. A stock solution of phospholipase A_2 was prepared from *N. naja siamensis* venom as described previously [8]; 1 ml of this preparation is equivalent to 1 mg of the original venom. This phospholipase A_2 preparation showed no proteolytic activity towards albumin, as measured by proton production monitored by a Radiometer TTT2 pH-stat autotitrator.

Mitochondria or submitochondrial vesicles were diluted to 10 mg protein per ml in 0.25 M sucrose, 10 mM dithiothreitol, 3 mM $CaCl_2$ and 25 mM glycylglycine (pH 7.4). This suspension was warmed to 37° C and the digestion started by the addition of an aliquot of the phospholipase A_2 preparation. Mitochondria and submitochondrial vesicles were digested with 4 and 5 μg, respectively, of phospholipase preparation per mg protein. Allowing for the mitochondrial matrix protein, which constitutes approx. 20% of the total protein of beef heart mitochondria, the ratio of phospholipase to membrane phospholipid was similar for the two preparations. At time intervals, 1-ml samples were removed and the lipolysis stopped by mixing with a small volume of cold, buffered EDTA, to give a final suspension of 8.6 mg protein per ml, 0.21 M sucrose, 5 mM dithiothreitol, 10 mM EDTA, 0.4 M LiBr and 20 mM PIPES-HCl (pH 6.5); the LiBr is required for the efficient solubilization of D-β-hydroxybutyrate dehydrogenase from the membrane [23]. The samples, digested for different times, were centrifuged in a Spinco 65 rotor; mitochondria at 40 000 rev./min for 20 min and submitochondrial vesicles at 55 000 rev./min for 40 min. The supernatants were decanted and assayed for D-β-hydroxybutyrate dehydrogenase activity following preincubation with added mitochondrial phospholipid, since the solubilized enzyme is inactive in the absence of added leci-

thin [24,25]. Mitochondrial supernatants were also assayed for glutamate and malate dehydrogenase activities. The pellets were resuspended in 1 ml of 0.25 M sucrose, 1 mM EDTA and 5 mM HEPES-HCl (pH 7.0) and each divided into two aliquots. One aliquot was assayed for protein, malate dehydrogenase and D-β-hydroxybutyrate dehydrogenase activities (in mitochondria) or for protein, D-β-hydroxybutyrate dehydrogenase, succinate-cytochrome *c* reductase and succinate oxidase activities (in submitochondrial vesicles). To the other aliquot was added 10% (w/v) albumin to a final concentration of 10 mg/ml. This second aliquot was washed to remove lysophosphatides and free fatty acids, produced during the digestion, using the serum albumin washing procedure described by Fleischer and Fleischer [18]. The washed samples, digested for varying times, were assayed without delay for D-β-hydroxybutyrate dehydrogenase activity, with and without the addition of mitochondrial phospholipid and for glutamate and malate dehydrogenase activities (in mitochondria) or for succinate-cytochrome *c* reductase and succinate oxidase activities (in submitochondrial vesicles). Each experiment was performed at least twice, one time with a larger quantity of material (50 mg of mitochondria or 25 mg of submitochondrial vesicles per time point) to allow more rapid handling of the samples during the albumin washing procedure.

The rate of lipolysis with phospholipase A_2 was monitored in a similar manner to the rate of proteolysis (see above) except that the pH was maintained at 7.4.

Results

We have compared the properties of D-β-hydroxybutyrate dehydrogenase in intact bovine heart mitochondria and in submitochondrial vesicles which have different faces of the membrane exposed. In mitochondria, which are 'right-side out', the cytochrome *c* face of the mitochondrial inner membrane is accessible to the medium and the matrix face is inaccessible. Submitochondrial vesicles are 'inside-out' so that the matrix face of the membrane is accessible to the medium and the cytochrome *c* face is inaccessible. Cytochrome *c* can accept electrons from substrate in mitochondria but not from submitochondrial vesicles where the cytochrome *c* face is the inner face and since cytochrome *c* cannot penetrate the mitochondrial inner membrane. The ratio of succinate oxidase to cytochrome *c* reductase in our bovine heart submitochondrial vesicles is 23 to 1 (Table I); this ratio is an expression that 95% or greater of the submitochondrial vesicles are 'inside-out'. Cytochrome *c* can penetrate the outer membrane of the bovine heart mitochondria used in this study since optimal succinate to cytochrome *c* reductase activity can be measured [22].

Submitochondrial vesicles exhibit optimum D-β-hydroxybutyrate dehydrogenase activity (varying between 0.4 and 0.54 μmol NAD^+ reduced/min per mg protein for different vesicle preparations) when assayed in either the standard assay medium (hypotonic) or in the presence of 0.25 M sucrose (isotonic). On the other hand, D-β-hydroxybutyrate dehydrogenase activity is not detected in mitochondria assayed in isotonic medium; some activity (approx. 0.20 μmol NAD^+ reduced/min per mg protein) can be measured in the hypotonic assay medium. Since NAD^+ is not readily permeable through the mitochondrial

TABLE I

THE RELEASE OF D-β-HYDROXYBUTYRATE DEHYDROGENASE FROM BOVINE HEART SUBMITOCHONDRIAL VESICLES BY PHOSPHOLIPASE A_2 DIGESTION

The vesicles were digested for the times indicated, as described in the text, followed by centrifugation to obtain the pellet and supernatant. D-β-hydroxybutyrate dehydrogenase activity and bound phosphorus were measured in the washed pellets. Succinate oxidation (succinate-cytochrome *c* reductase and succinate oxidase) was measured in the unwashed pellets.

Digestion time (min)	Bound phosphorus (μg/mg protein)	D-β-Hydroxybutyrate dehydrogenase (μmol NAD^+ reduced/min per mg)		Succinate oxidation (μmol (2*e*)/min per mg)		
		Pellet	Supernatant	→Cytochrome *c*	→O_2	O_2/Cytochrome *c*
Original	22	0.40	—	0.048	1.11	23.1
0	22	0.39	0	0.071	1.66	23.4
1	19	0.23	0.04	0.070	1.61	23.0
2	18	0.09	0.12	0.080	1.49	18.6
3	16	0.06	0.15	0.110	1.24	11.3
4	15	0.04	0.14	0.300	1.32	4.4
5	14	0.02	0.14	0.444	1.07	2.4
6	14	0.01	0.14	0.798	1.09	1.4
10	11	0	0.12	0.834	1.19	1.4

inner membrane, the latency of enzymic activity suggests that the cofactor binding site of D-β-hydroxybutyrate dehydrogenase is localized on the matrix face of the mitochondrial inner membrane.

Digestion of mitochondria and submitochondrial vesicles with trypsin

D-β-Hydroxybutyrate dehydrogenase activity in submitochondrial vesicles is considerably more labile to trypsin digestion than the enzyme in intact mitochondria (Fig. 1). Using comparable conditions, D-β-hydroxybutyrate dehydrogenase activity was completely inactivated in 15 min in submitochondrial vesicles, whereas, greater than 95% of the activity remains in the mitochondria. We have also demonstrated, using the pH-stat to titrate the protons produced during digestion, that the rate of proteolysis with trypsin was the same for both the mitochondria and submitochondrial vesicle preparations. Therefore, D-β-hydroxybutyrate dehydrogenase has a trypsin-labile site which is exposed on the outer surface of the submitochondrial vesicles. In intact mitochondria, this site is not readily accessible to trypsin.

Digestion of mitochondria with phospholipase A_2

Freshly prepared bovine heart mitochondria were digested for various times with phospholipase A_2 and the membranes separated from the solubilized proteins by centrifugation. The activities of D-β-hydroxybutyrate dehydrogenase and malate dehydrogenase (a matrix enzyme) were measured in the supernatant and in the resuspended pellets to determine the rates of release of these enzymes from the organelle. The results of a typical experiment are shown in Fig. 2. Malate dehydrogenase activity is lost from the pellet at about the same rate or slightly more rapidly than is D-β-hydroxybutyrate dehydrogenase,

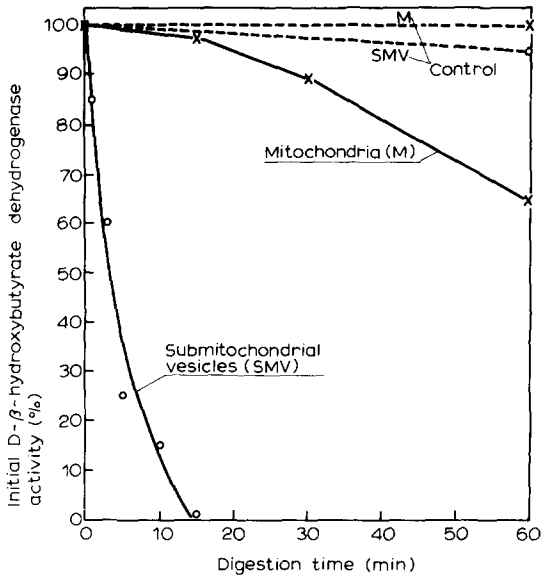

Fig. 1. The inactivation of D-β-hydroxybutyrate dehydrogenase in bovine heart mitochondria (X) or submitochondrial vesicles (○) by digestion with trypsin. The membrane preparations (2 mg protein/ml) were incubated for the times indicated, at room temperature (25°C), with trypsin (5%, by weight) and enzymic activity was measured after varying times of digestion. The initial specific activities of the mitochondria assayed in hypotonic medium and the submitochondrial vesicles were, respectively, 0.23 and 0.56 μmol NAD^+ reduced/min per mg protein. The solid lines are samples with trypsin digestion and the dashed lines are controls without trypsin addition.

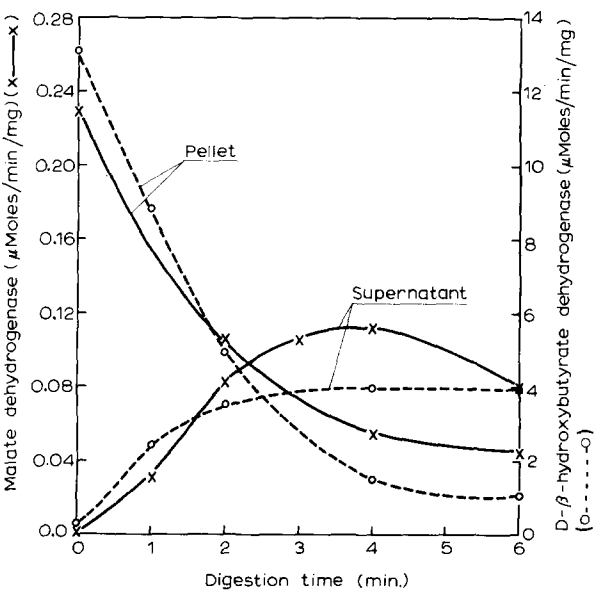

Fig. 2. The release of D-β-hydroxybutyrate dehydrogenase and malate dehydrogenase from bovine heart mitochondria by phospholipase A_2 digestion. Mitochondria (10 mg/ml) were digested for the times indicated, at 37°C, with phospholipase A_2 (4 μg/mg protein) as described in the text. D-β-hydroxybutyrate dehydrogenase and malate dehydrogenase activities were measured in the pellets and supernatants obtained by centrifugation.

which suggests that D-β-hydroxybutyrate dehydrogenase is released from the mitochondrial membrane concomitant with or following the release of the soluble matrix proteins (as monitored by malate dehydrogenase release). The loss of glutamate dehydrogenase activity from the mitochondrial pellets followed a time course similar to that of malate dehydrogenase (data not shown).

The rate of appearance of D-β-hydroxybutyrate dehydrogenase and malate dehydrogenase in the supernatant (Fig. 2) also indicates that malate dehydrogenase is relased by phospholipase digestion at a similar rate or even more rapidly than the solubilization of D-β-hydroxybutyrate dehydrogenase, although only a portion of the activity of each enzyme is recovered in the supernatant. D-β-Hydroxybutyrate dehydrogenase is bound to the mitochondrial inner membrane, whereas malate dehydrogenase is localized in the matrix space. The results presented here are compatible with the interpretation that D-β-hydroxybutyrate dehydrogenase is localized on the matrix face of the mitochondrial inner membrane.

Digestion of submitochondrial vesicles with phospholipase A_2

Submitochondrial vesicles were digested with phospholipase A_2, using approximately the same weight ratio of phospholipase to membrane protein as was used with intact mitochondria. The loss of D-β-hydroxybutyrate dehydrogenase from the membrane and solubilization into the supernatant is shown in Table I; only 35% of the membrane-bound activity is obtained in the supernatant, which is similar to the amount obtained from mitochondria. The rate of lipolysis, measured by pH-stat titration of the free fatty acid produced, was shown to be the same for submitochondrial vesicles as for mitochondria. However, release of D-β-hydroxybutyrate dehydrogenase from the vesicle membranes is more rapid than from intact mitochondria, i.e. half of the membrane-bound activity is released from the vesicles in approx. 1 min; whereas, 2 min digestion is required for the release of 50% of the enzyme from mitochondria (Fig. 2). The maximum activity for the solubilized enzyme is obtained between 2 and 3 min of digestion of the submitochondrial vesicles but 4—6 min of digestion is required for maximum activity to be obtained in the supernatant from mitochondria. The more rapid release of D-β-hydroxybutyrate dehydrogenase from submitochondrial vesicles than from freshly-prepared, intact mitochondria, is also compatible with the view that the enzyme is localized on the outer face of the vesicle preparation, i.e. the matrix face of the mitochondrial inner membrane.

More compelling evidence for the localization of D-β-hydroxybutyrate dehydrogenase on the matrix face of the mitochondrial inner membrane was obtained by measuring the rate of release of the enzyme from submitochondrial vesicles and correlating this release with the permeability of the membrane to cytochrome *c* (as measured by succinate-cytochrome *c* reductase activity) with increasing phospholipase A_2 digestion time. Submitochondrial vesicles have a low succinate-cytochrome *c* reductase activity because the inner surface, which transfers electrons to cytochrome *c* is not accessible to the cytochrome *c* in the medium. When submitochondrial vesicles are digested extensively with phospholipase A_2, the membrane becomes leaky to cytochrome *c*; whereas the succinate oxidase activity is not markedly altered. Thus the oxidase/reductase

ratio (initially 23) is reduced to 1.4 when the membrane is made leaky by phospholipase A_2 digestion (Table I). Under the conditions of the experiment, 85% of the D-β-hydroxybutyrate dehydrogenase activity is lost from the pellet and optimum activity obtained in the supernatant at 3 min of digestion. At this time of digestion the succinate to cytochrome *c* reductase activity has increased to only 0.11 μmol cytochrome *c* reduced/min per mg protein; this activity is equivalent to 13% of the vesicles being leaky to cytochrome *c*, taking the succinate to cytochrome *c* reductase activity (0.83 μmol cytochrome *c* reduced/min per mg protein), obtained at 10 min of digestion, to represent 100% of the vesicles being permeable to cytochrome *c*. This is a conservative estimate for the percentage of vesicles that are leaky to cytochrome *c* at 3 min of digestion since the succinate to cytochrome *c* reductase activity at 10 min of digestion is lower than that expected for fully opened vesicles, i.e. the oxidase/reductase ratio is 1.4 whereas the theoretical ratio would be 1.0. D-β-Hydroxybutyrate dehydrogenase activity is lost from the pellet and optimum activity of the enzyme is obtained in the supernatant before the cytochrome *c* reductase activity becomes markedly increased. It should also be noted that submitochondrial vesicles, which have been treated with phospholipase A_2 for 3 min to release D-β-hydroxybutyrate dehydrogenase, have no breaks or discontinuities in their membranes and appear indistinguishable from the original vesicles, as observed by electron microscopy (not shown).

Thus, D-β-hydroxybutyrate dehydrogenase is released from the membrane before the submitochondrial vesicles become leaky to cytochrome *c*. Since cytochrome *c* (12 300 daltons), which is considerably smaller than the D-β-hydroxybutyrate dehydrogenase monomer (31 300 daltons), cannot enter the vesicles, D-β-hydroxybutyrate dehydrogenase, if it were on the inside, could not get out. Hence the D-β-hydroxybutyrate dehydrogenase, which was released, must have been on the outside (matrix face) on the submitochondrial vesicles. No more enzyme was released by prolonged digestion. We conclude that D-β-hydroxybutyrate dehydrogenase is localized on the matrix face of the mitochondrial inner membrane.

Discussion

This study is concerned with the sidedness and orientation of D-β-hydroxybutyrate dehydrogenase in the membrane. The enzyme has been shown definitively to be localized on the matrix face of the mitochondrial inner membrane.

Suggestive evidence that D-β-hydroxybutyrate dehydrogenase is localized on the matrix face has been obtained by comparing beef heart mitochondria with well-defined inside-out submitochondrial vesicles. We have shown that: (1) the activity is latent in mitochondria and optimal in submitochondrial vesicles; (2) the enzyme is more rapidly released from submitochondrial vesicles by phospholipase A_2 digestion than from mitochondria (Fig. 2 and Table I); and (3) the enzyme is very labile to proteolytic digestion in submitochondrial vesicles as compared with mitochondria (Fig. 1), even though the extent of proteolysis was similar in both cases.

Previous studies with whole mitochondria support this interpretation. Lehninger et al. [26] reported that D-β-hydroxybutyrate dehydrogenase activ-

ity was not observed in intact rat liver mitochondria and was expressed only after disruption of the organelle. Gaudemer and Latruffe [27] found that permeant rather than impermeant thiol reagents inactivated D-β-hydroxybutyrate dehydrogenase in rat liver mitochondria. In the approaches referred to above, both from other laboratories as well as our own, the evidence regarding the sidedness of D-β-hydroxybutyrate dehydrogenase in the mitochondrial membrane is not definitive since mitochrondria and submitochondrial vesicles differ in size and complexity as well as in sidedness.

We have obtained conclusive evidence by studying the release of D-β-hydroxybutyrate dehydrogenase from submitochondrial vesicles by phospholipase A_2 digestion. The enzyme is released from the vesicles which remain impermeable to cytochrome *c* (Table I). Thus, D-β-hydroxybutyrate dehydrogenase must be localized on the outside of the submitochondrial vesicle which is equivalent to the matrix face of the mitochondrial inner membrane.

A great deal of experience has accumulated regarding the nature of membrane proteins and their orientation in membranes. It is appropriate and timely to consider the possible ways that proteins can be oriented in the phospholipid bilayer and to categorize D-β-hydroxybutyrate dehydrogenase within this framework.

Selective extraction of membranes with dilute acid or urea gave rise to the notion that some proteins designated 'membrane-associated proteins' were more readily releasable than others which remain intimately associated with the membrane superstructure, i.e. the trilayer [2,3]. The extracted residue which was membranous as viewed by electron microscopy, contained the intrinsic proteins and all of the lipid. Special fixation allowed the visualization of the membrane-associated protein as a 'surface fuzz'. Later, other terms such as 'extrinsic versus intrinsic' [28] and 'peripheral versus integral' [1] were introduced.

Membrane proteins are either intimately associated with the phospholipid bilayer (intrinsic proteins) or associated at the surface (membrane-associated proteins). There are four basic modes of protein orientation in the membrane (Fig. 3), three of these are intrinsic. (1) Transmembrane proteins are bipolar and span the membrane, connecting both aqueous compartments. (2) Inlaid proteins are amphipathic or monopolar and as such are partially inserted into a hydrophobic portion of the bilayer with the polar end in contact with the aqueous phase. The hydrophobic portion of the amphipathic molecule serves as an anchor while the polar segment, which protrudes into the aqueous compartment, can interact with substrate or the functional groups of other membrane proteins or with soluble enzymes. (3) Buried proteins are hydrophobic or nonpolar and are immersed within the hydrophobic milieu of the lipid with no access to either aqueous compartment. (4) Membrane-associated proteins are less firmly associated with the membrane as compared with intrinsic proteins (Nos. 1—3, above). They reside in the aqueous phase and can be attached to the membrane superstructure either via phospholipid by polar association or via intrinsic protein or via both phospholipid and protein.

These orientations are illustrated diagrammatically in Fig. 3. The polar segment of the transmembrane or inlaid proteins could be extensive or, in the limit, flush with the hydrophilic surface of the bilayer. Thus, a further distinction can

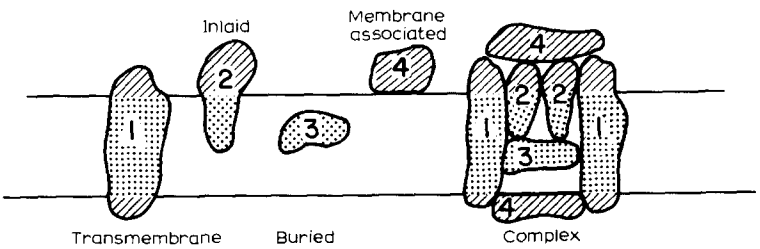

Fig. 3. A diagrammatic representation of the possible ways in which proteins can be oriented in the membrane (see text). The proteins are visualised as being inserted into a phospholipid bilayer. The dotted and cross-hatched areas indicate, respectively, the hydrophobic and hydrophilic surfaces of the proteins. The four basic modes of orientation of membrane proteins, designated by the numbers (1—4), are transmembrane (1), inlaid (2), buried (3) intrinsic proteins and membrane-associated (4) proteins. Examples of transmembrane proteins include a variety of transport proteins and exchange transporters. Cytochrome b_5 and NADH-cytochrome b_5 reductase of endoplasmic reticulum [31] exemplify the inlaid orientation. Proteolipid of myelin [32] or subunit 9 of ATP synthetase [33] are examples of buried hydrophobic proteins. The three types of interaction of membrane-associated proteins are (a) lipid associated, e.g. cytochrome *c* bound to phospholipid vesicles containing acidic lipids [34]; (b) protein associated, e.g. spectrin in the red blood cell [35]; and (c) lipid-protein associated, e.g. cytochrome *c* in mitochondria [36]. The 'complex' contains more than one type of subunit. The one shown at the right has protein components which are representative of the four basic orientations, including two types of membrane-associated protein (4). The upper one is associated with protein only whereas the lower one is associated with both phospholipid and protein. The ATP synthetase of mitochondria is an example of a membrane complex.

be made based on whether the polar segment does or does not protrude from the membrane surface into the aqueous domain, so that transmembrane proteins can have either none (for example, bacteriorhodopsin of the purple membrane of *Halobacterium halobium* [29]), one (for example, Ca^{2+} pump protein of sarcoplasmic reticulum [46]) or both of the polar groups (for example, glycophorin of the red blood cell membrane [30]) extending into the aqueous compartment. Variations of the basic types of intrinsic protein can be envisioned. For instance, a bipolar molecule could be oriented in the membrane in a cis configuration with both polar ends facing or extending into the same aqueous compartment and connected by a single hydrophobic segment in the hydrophobic interior of the bilayer. This would be a variant of an amphipathic molecule.

Most membrane proteins are multimers, i.e. composed of subunits. The Ca^{2+} pump protein of sarcoplasmic reticulum is a simple oligomer, composed of 3 or 4 protomers of a single type of subunit [37,38]. The term complex is suggested to designate multimers, composed of more than one type of subunit, each of which could be oriented in the membrane in any of the four basic modes. The possible diversity in orientation and properties of the individual components of a complex must be taken into account when the role of each component in the function and organization of the complete complex is considered. The ATP synthetase of mitochondria is an example of a complex multimer composed of several intrinsic and membrane-associated proteins [40].

Mitochondrial D-β-hydroxybutyrate dehydrogenase is present in different tissues in a broad range of species [26] with the notable exception of ruminant liver mitochondria [40]. It is one of the best documented and most intensively studied lipid-requiring enzymes. In view of its absolute phospholipid require-

ment for function, the enzyme in the membrane must be intimately associated with phospholipid. In addition, D-β-hydroxybutyrate dehydrogenase cannot be removed from the membrane by washing with chelators or varying concentrations of salts. This strong interaction of the enzyme with the membrane superstructure together with the phospholipid requirement indicate that D-β-hydroxybutyrate dehydrogenase is an intrinsic membrane protein.

Our studies suggest that D-β-hydroxybutyrate dehydrogenase has an inlaid rather than a transmembrane orientation in the mitochondrial inner membrane. The release of an amphipathic molecule from its orientation in the membrane, would be more favorable energetically, than the release of a transmembrane protein, which is bipolar. The release of transmembrane proteins generally requires the complete solubilization of the membrane [12]. It is the general experience that partial digestion of membranes with phospholipase A_2 does not release transmembrane proteins [18,41,42]. D-β-Hydroxybutyrate dehydrogenase is released from submitochondrial vesicles when only 27% of the phospholipid is degraded with phospholipase A_2 and while the membrane remains intact morphologically and impermeable to cytochrome *c*. The characteristics of this release are compatible with an inlaid orientation. We have recently shown that purified D-β-hydroxybutyrate dehydrogenase can readily be inserted into membranes and when rebound is functional [43]. The release and insertion characteristics combined strongly suggest that D-β-hydroxybutyrate dehydrogenase is inlaid in the membrane. The functional form of D-β-hydroxybutyrate dehydrogenase appears to be a dimer [45]. From the above considerations, the dimer is amphipathic and inserted into the matrix face of the mitochondrial inner membrane in an inlaid orientation.

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