Monoclonal antibodies for structure–function studies of (*R*)-3-hydroxybutyrate dehydrogenase, a lipid-dependent membrane-bound enzyme

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Monoclonal antibodies (mAbs) have been used to study structure–function relationships of (*R*)-3-hydroxybutyrate dehydrogenase (BDH) (EC 1.1.1.30), a lipid-requiring mitochondrial membrane enzyme with an absolute and specific requirement for phosphatidylcholine (PC) for enzymic activity. The purified enzyme (apoBDH, devoid of phospholipid and thereby inactive) can be re-activated with preformed phospholipid vesicles containing PC or by short-chain soluble PC. Five of six mAbs cross-react with BDH from bovine heart and rat liver, including two mAbs to conformational epitopes. One mAb was found to be specific for the C-terminal sequence of BDH and served to: (1) map endopeptidase cleavage and epitope sites on BDH; and (2) demonstrate that the C-terminus is essential for the activity of BDH. Carboxypeptidase cleavage of only a few ($\leqslant 14$) C-terminal amino acids from apoBDH (as detected by the loss of C-terminal epitope for mAb 3-10A) prevents activation by either bilayer or soluble PC. Further, for BDH in bilayers containing PC, the C-terminus is protected from carboxypeptidase cleavage, whereas in bilayers devoid of PC the *C*-terminus is cleaved, and subsequent activation by PC is precluded. We conclude that: (1) the C-terminus of BDH is essential for enzymic activity, consistent with the prediction, from primary sequence analysis, that the PC-binding site is in the C-terminal domain of BDH; and (2) the allosteric activation of BDH by PC in bilayers protects the C-terminus from carboxypeptidase cleavage, indicative of a PC-induced conformational change in the enzyme.

INTRODUCTION

(*R*)-3-Hydroxybutyrate dehydrogenase (BDH; EC 1.1.1.30) is a lipid-requiring enzyme which has a specific requirement for phosphatidylcholine (PC) for activity. It is localized at the matrix face of the mitochondrial inner membrane (McIntyre et al., 1978a; Fleischer et al., 1983). The enzyme has been purified from bovine heart and rat liver mitochondria (Bock and Fleischer, 1974; McIntyre et al., 1988) and more recently from rat brain (Zhang and Churchill, 1990). Purified BDH is devoid of lipid or detergent and is referred to as apoBDH. It is soluble in dilute aqueous solution (Nielsen and Fleischer, 1973; McIntyre et al., 1978b) and is essentially inactive in the absence of added phospholipids. BDH consists of a single subunit (molecular mass approx. 31.5 kDa) (Bock and Fleischer, 1975) and, as determined by target inactivation analyses, is tetrameric in native membranes [submitochondrial vesicles (SMV)] and when reconstituted with phospholipid vesicles (McIntyre et al., 1983). ApoBDH inserts spontaneously and unidirectionally into preformed phospholipid vesicles or natural membranes (McIntyre et al., 1979), but optimal activation of the enzyme is obtained only with membranes containing PC (Gazzotti et al., 1975; Latruffe et al., 1986). Activation is specific for the phosphocholine moiety of PC (Isaacson et al., 1979), but bilayer association of BDH is not essential, since monomeric short-chain PC can also activate BDH (Gazzotti et al., 1975; Cortese et al., 1989). Activation of BDH by bilayer PC appears to involve an allosteric mechanism (Sandermann et al., 1986) in which PC enhances binding of nucleotide by more than an order of magnitude (Rudy et al. 1989).

The primary sequences of the enzymes from human heart (Marks et al., 1992) and rat liver (Churchill et al., 1992) have recently been determined by cDNA cloning. Analysis of the predicted amino acid sequence of human heart BDH indicates that the C-terminal third of the sequence probably contains elements that determines the substrate specificity of the enzyme including the phospholipid (including PC)-binding site(s) (Marks et al., 1992). A number of previous studies indicate that the bilayer topology of BDH differs from the enzyme reconstituted with phospholipid vesicles with or without PC. Treatment with chaotropic LiBr can dissociate BDH from vesicles only in the absence of PC (Gazzotti et al., 1975), and spectroscopic studies (Dalton, 1990) indicate that a reactive cysteine is located deeper within the bilayer in the presence of PC. Also, apoBDH and BDH reconstituted with phospholipid vesicles lacking PC are sensitive to a number of endo- and exo-peptidases, whereas BDH

Abbreviations used: BDH, (*R*)-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30); apoBDH, purified BDH devoid of lipid or detergent; BDH–MPL, the complex of BDH reconstituted with vesicles of mitochondrial phospholipids; DPG, diphosphatidylglycerol; DTT, dithiothreitol; mAb, monoclonal antibody; MPL, total mitochondrial phospholipids; PC, phosphatidylcholine; $PC(8:0)_2$, dioctanoyl-PC; PE, phosphatidylethanolamine; SMV, submitochondrial vesicles; TBS, 10 mM Tris/HCl, pH 8.1, 0.1 M NaCl; TBST, TBS containing 0.05% (v/v) Tween 20; TBST0.5, TBST with a total of 0.5 M NaCl.

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either in the mitochondrial membrane (in SMV) or purified and reconstituted with PC-containing membranes is generally resistant to proteases (Berrez et al., 1984; Maurer et al., 1985). Thus the orientation of BDH in the bilayer appears to be modulated by the presence of PC, and this may reflect the activation of the enzyme by PC.

Monoclonal antibodies (mAbs) have been used to probe the topology of a number of membrane proteins, such as *lac* permease (Kaback, 1986) and the mitochondrial ATP synthase (Moradi-Ameli and Godinot, 1987). In this study, mAbs against BDH from bovine heart and rat liver have been developed as probes for topological studies of the enzyme. A key finding is that C-terminal amino acids are essential for enzyme function and activation by PC. A preliminary report of portions of this work has appeared (Adami et al., 1990).

EXPERIMENTAL

Routine analytical procedures

Protein was assayed by the method of Lowry et al. (1951) as described by Rudy et al. (1989). Phospholipids and membranes were assayed for total phosphorus by the method of Rouser and Fleischer (1967). SDS/PAGE was performed either as described by Laemmli (1970) or, for samples subjected to partial proteolysis, with the Tris/Tricine buffer system of Schägger and Von Jagow (1987); *m*-Cresol Purple was used as the tracking dye, as it migrates faster than peptides of approx. 2 kDa (Christy et al., 1989). Gel dimensions were 7 cm × 8 cm (length × width) and 0.75 mm thick. Protein molecular-mass standards for SDS/PAGE were either from BRL (Gaithersburg, MD, U.S.A.) [3–43 kDa as follows: insulin 3 kDa (apparent size); bovine trypsin inhibitor, 6.2 kDa; lysozyme, 14.3 kDa; β-lactoglobulin, 18.4 kDa; carbonic anhydrase, 29 kDa; and ovalbumin, 43 kDa] or from Bio-Rad (Richmond, CA, U.S.A.) (14.3–97.4 kDa as follows: lysozyme, 14.3 kDa; soyabean trypsin inhibitor, 21.5 kDa; bovine carbonic anhydrase, 31 kDa; ovalbumin, 43 kDa; BSA, 66 kDa; and rabbit muscle phosphorylase *b*, 97 kDa). Prestained SDS/PAGE protein standards were from Bio-Rad. Gels were stained with Coomassie Brilliant Blue or silver (Merill et al., 1983) or were used for Western blotting as described below.

Preparation of membranes, enzymes and phospholipid vesicles

SMV from bovine heart and rat liver were isolated from mitochondria as described by Fleischer et al. (1974). Bovine heart and rat liver BDHs were purified from the respective mitochondria as described (McIntyre et al., 1988). Stocks of apoBDH were stored in buffer containing 5 mM Hepes/NaOH, pH 7.5, 5 mM dithiothreitol (DTT) and LiBr (0.15 M for the rat liver enzyme and 0.4 M for the bovine heart enzyme). Phospholipid vesicles [PC, phosphatidylethanolamine (PE)/diphosphatidylglycerol (DPG) (9:1, by phosphorus), PC/PE/DPG (5:4:1, by phosphorus) or mitochondrial phospholipids (MPL)] were prepared by bath sonication (Rouser and Fleischer, 1967; Sandermann et al., 1986) and were filtered (Millipore GSTF 0.22 μM). PC and PE (both dioleoyl) and bovine mitochondrial DPG were from Avanti Polar Lipids (Pelham, AL, U.S.A.).

Reconstitution of BDH with phospholipid vesicles and membranes

The method of Churchill et al. (1983a) for preparing BDH–vesicle complexes was used for most purposes. Briefly, samples of apoBDH stock solution (typically 1–2 mg/ml) were diluted to 0.1–0.25 mg/ml in buffer (20 mM Tris/HCl, 1 mM EDTA, 5 mM DTT, pH 8.1) containing preformed phospholipid vesicles ($\geqslant$ 150 μg of P/mg of BDH). These samples were incubated at 30 °C for 1–1.5 h to optimally activate BDH (see McIntyre et al., 1979; Churchill et al., 1983a).

For some experiments in which apoBDH was incubated with an antibody or protease, a portion of apoBDH was subsequently reconstituted with PC/PE/DPG vesicles (5:4:1, by phosphorus) in a cuvette in order to assay for BDH activity (see below). For these reconstitutions, the cuvette contained the buffer and NAD^+ plus an excess of PC/PE/DPG vesicles (800–1000 mol of phospholipid/mol of BDH). After addition of the apoBDH sample (0.5 or 1 μg in $\leqslant$ 5 μl), the cuvette was incubated for 15 min (at 37 °C) to optimally re-activate BDH before initiation of the enzyme assay with (*R*,*S*)-3-hydroxybutyrate.

Assays of BDH activity

BDH activity was measured in a phosphate-buffered cocktail, essentially as described previously (Bock and Fleischer, 1975; Rudy et al., 1989) using 10 mM NAD^+ and 20 mM (*R*,*S*)-3-hydroxybutyrate (unless noted otherwise) and at either 30 °C or 37 °C. BDH activity in SMV was measured in the presence of 0.5 μg/ml antimycin A to prevent oxidation of NADH by the electron-transport system. For activation of aqueous BDH by monomeric dioctanoyl-PC [$PC(8:0)_2$; Avanti Polar Lipids], assays followed a previous procedure (Cortese et al., 1982), but were carried out at 30 °C, in a solution containing 50 mM Hepes, 1 mM EDTA, 1 mM DTT and 5% (v/v) ethanol, pH 7.4, to optimize re-activation (T.M. Duncan, unpublished work).

Limited proteolytic digestion of apoBDH in the presence of SDS

Buffers for performing and stopping proteolysis were as described by Cleveland (1983), and endopeptidase stocks were prepared in 0.125 M Tris/HCl, pH 6.8 (at 24 °C). Proteolytic digestions were performed at room temperature for 10–30 min and typically contained 0.12 mg of apoBDH/ml and one of the proteases at a typical concentration: α-chymotrypsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.), 25 μg/ml; glutamate-specific endopeptidase SV8 (Boehringer–Mannheim Biochemicals, Indianapolis, IN, U.S.A.), 1 μg/ml; lysine-specific endopeptidase (Boehringer–Mannheim), 6 μg/ml. To stop proteolysis, samples were diluted with an equal volume of solubilization mixture (Cleveland, 1983) containing 0.01% *m*-Cresol Purple and immediately heated at 95 °C for 2–4 min, then analysed by SDS/PAGE as described above.

Cleavage of BDH with carboxypeptidase Y

Carboxypeptidase Y (Boehringer–Mannheim) was dissolved in 100 mM Tris/HCl, pH 6.8. BDH–phospholipid complexes in the complex reconstitution buffer (10 mM Tris/HCl, pH 8.1, 15 mM LiBr, 1 mM EDTA, 5 mM DTT) were treated with carboxypeptidase Y (usually 2.0 or 2.5%, w/w). ApoBDH was cleaved in a solution containing 10 mM Tris/HCl, pH 8.1 (at 24 °C), 150 mM LiBr, 1 mM EDTA, 5 mM DTT and 20% (w/v) glycerol (Maurer et al., 1985) or, for testing the effect of $PC(8:0)_2$ on cleavage, in a solution containing 50 mM Hepes, 1 mM EDTA, 1 mM DTT and 5% (v/v) ethanol, pH 7.4. Digestion with carboxypeptidase Y was stopped by adding aprotinin (United States Biochemical Corp., Cleveland, OH, U.S.A.) to 20 μg/ml and, in some experiments, 0.5 mM phenylmethanesulphonyl fluoride.

Photodensitometry

Digitized images of stained gels, Western blots and dot blots were produced with a diode-array-based photodensitometric camera system (Technology Resources, Nashville, TN, U.S.A.). The system software gave reproducible determination of R_F values for BDH proteolytic fragments on stained gels and Western blots; molecular-mass values were determined from regression fits of R_F values for protein molecular-mass standards (obtained both from Promega, Madison, WI, U.S.A. and Gibco-BRL, Gaithersburg, MD, U.S.A.). For dot-blots, entire dotted sample areas were measured to quantify the relative dot-blot intensities; in most cases, samples applied were adjusted to give absorbance values within a range in which known amounts of BDH produced a linear response.

Immunochemical methods

Generation of mAbs against BDH

Hybridomas producing mAbs were prepared by standard methods using Balb/c mice (Davis, 1986; Fu and Carter, 1990). For presentation as antigen, purified BDH (approx 2.5 mg of protein/ml) was dialysed against a solution containing 0.1 M NaCl, 2 mM Hepes (pH 7.0) and 0.5 mM DTT and then either diluted directly into PBS (native enzyme in 0.2 ml, 10 μg of bovine heart BDH or 20 μg of rat liver BDH) or diluted after heat denaturation (95 °C in 2% SDS; 20 μg of bovine heart BDH in 0.2 ml). The enzyme was emulsified with an equal volume of Freund's complete adjuvant and each mouse was injected subcutaneously (for bovine heart BDH antigens) or intraperitoneally (rat liver BDH antigens). Each mouse was boosted with the same amount of antigen, emulsified with Freund's incomplete adjuvant, after 2, 3 and 4 weeks (bovine heart BDH) or after 2 and 4 weeks (rat liver BDH). One week after the last boost with antigen, mice with the highest serum titre as measured by e.l.i.s.a. (see below) were selected for mAb production and were injected in the tail vein with 10 μg of antigen in 50 μl of saline either 1 (rat liver BDH) or 3 (bovine heart BDH) days before the animals were killed (day 40 or 41). To prepare anti-(bovine heart BDH) mAbs, spleen cells were prepared from two mice (one from each group immunized with native or denatured antigen). Spleen cells from each mouse were fused with Balb/c-derived SP2 murine myeloma cells using 45% poly(ethylene glycol) 8000. Hybridomas were selected in a standard hypoxanthine/aminopterin/thymidine medium (RPMI 1640 from Gibco, Grand Island, NY, U.S.A.), and positive clones were identified by e.l.i.s.a. with purified BDH (native or denatured) as antigen. Positive clones were expanded and subcloned two to three times to obtain monoclonal cultures.

For anti-(bovine heart BDH) mAbs, a number of independent clones were injected intraperitoneally (approx 2×10^6 hybridoma cells) into pristane-primed Balb/c mice. Within 2 weeks, ascites fluid was removed with a syringe and the cells were removed by centrifugation. For most studies described here, the mAbs in ascites fluid were used without additional purification. For some experiments, the mAbs were partially purified by $(NH_4)_2SO_4$ fractionation [precipitation with 50% $(NH_4)_2SO_4$ in 0.25 M Tris/HCl, pH 8.0, for 1 h at 4 °C]. For anti-(rat liver BDH) mAbs, hybridoma cells were grown in large volumes and mAbs were isolated from the culture supernatants. For mAb 36-4-1, the immunoglobulin fraction was purified by chromatography on Protein A–Sepharose (McKenzie et al., 1978). The anti-(rat liver BDH) mAb 16-7-1 is IgM type and was purified from culture supernatant by hydroxyapatite chromatography as described by Stanker et al. (1985).

E.l.i.s.a.

Mouse sera, hybridoma supernatants and ascites fluids were screened for antibody activity by e.l.i.s.a. (Engvall, 1980) as described (Fu and Carter, 1990) using microtitre plates (96-well Immulon 2 plates; Dynatech Laboratories, Alexandria, VA, U.S.A.) coated with either native or denatured bovine heart BDH or native rat liver BDH (0.1 μg per well in 0.1 ml of 0.1 M carbonate buffer, pH 9.5, for 1 h at 37 °C and overnight at 4 °C). The immunoglobulin classification of each mAb was determined by e.l.i.s.a. with an immunoglobulin subtype-specific kit from Zymed Laboratories (San Francisco, CA, U.S.A.).

Dot-blot assays (Hawkes, 1986) of BDH

ApoBDH was diluted in TBS buffer (10 mM Tris/HCl, pH 8.1, 0.1 M NaCl) and 100 μl portions containing from 1 ng to 1 μg of BDH were applied as dots (area 32 mm^2) to a nitrocellulose membrane (0.45 μm) using a 96-well dot-blot apparatus (Schleicher and Schuell, Keene, NH, U.S.A.). The nitrocellulose was subjected to the following sequential incubations at room temperature: (i) 1 h with TBST buffer [TBS with 0.05% (v/v) Tween 20] containing 5% (w/v) non-fat milk protein to block non-specific protein binding; (ii) 2 h with the appropriate mAb [diluted in TBST + 1% (w/v) BSA]; (iii) three times for 5 min each in TBST0.5 (TBST with 0.5 M NaCl); (iv) 30 min with an anti-(mouse IgG) antibody–alkaline phosphatase conjugate (Promega), diluted approx. 1:6000 in TBST followed by washing as in step (iii); (v) in 0.1 M Tris/HCl, pH 9.4, 150 mM NaCl, 5 mM $MgCl_2$ (alkaline phosphatase buffer) containing 0.33 mg/ml 4-Nitro-Blue Tetrazolium chloride and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl phosphate; and (vi) with several changes of water to stop the colour development.

Western-blot analysis

Immediately after electrophoresis, proteins were transferred to a poly(vinylidene difluoride) membrane (Immobilon-P, 0.45 μm; Millipore Corp., Bedford, MA, U.S.A.) by the electrotransfer method of Towbin et al. (1979); 250 mA of constant current was applied for 1 h at room temperature, with a buffer composed of 25 mM Tris, 196 mM glycine, 0.005% (w/v) SDS and 15% (v/v) methanol. These transfer conditions were empirically determined to give essentially quantitative transfer of BDH and most proteolytic fragments of BDH (not shown). Subsequent incubations of the Immobilon membrane for immuno-detection were as described above for dot blots. In some cases, the TBST0.5 buffer used for washes contained 0.5% Tween 20 to reduce non-specific binding of antibodies further. Protein molecular-mass standards were run in at least two lanes of each blot to determine the R_F values of proteolytic fragments. After transfer to the Immunobilon-P membrane, the lanes with standard were cut from the blot and stained with Amido Black (Pluskal et al., 1986). The R_F values for antibody-reactive peptides on Western blots reported in these studies corresponded to the R_F values of peptides detected on duplicate Coomassie- or silver-stained gels. Molecular-mass values for the peptide fragments of BDH were estimated from the R_F values and plots of R_F versus log (molecular mass) for the protein standards.

Immunoprecipitation of BDH from SMV

Bovine heart SMV (0.5 mg) were incubated for 2 h at room temperature (about 24 °C) with mAb (20 μl of ascites fluid) in a total volume of 0.1 ml of TBST buffer containing 1% BSA; the membranes were then pelleted in a TL-100 ultracentrifuge

(220000 *g* for 10 min at 4 °C). The supernatants (containing unbound mAb) were discarded and each pellet of SMV with bound mAb was solubilized in 0.5 ml of TBST containing 1% Triton X-100 and re-centrifuged to remove unsolubilized membranes. The solubilized samples were diluted 4-fold with TBST, and the BDH–mAb complexes were immunoprecipitated with 60 μl of Protein G–Sepharose [approx 50% (v/v) in TBST] (Pharmacia, Piscataway, NJ, U.S.A.). Immunoprecipitates were washed five times (1 ml each) with TBST, and proteins were then eluted in 40 μl of sample buffer [2% (w/v) SDS in 60 mM Tris/HCl (pH 6.8), 5% 2-mercaptoethanol and 10% (v/v) glycerol containing 0.2 μg/ml *m*-Cresol Purple] for analysis by SDS/PAGE and Western blotting with anti-(bovine heart BDH) polyclonal antibodies.

RESULTS

Characterization of mAbs against BDH

Basic characteristics of six mAbs produced in this study are summarized in Table 1. All four mAbs produced against bovine heart BDH are the IgG_1 type and one of the mAbs produced against rat liver BDH is IgM type. Five of the six mAbs show cross-species reaction with BDH, consistent with the similarity of bovine heart and rat liver BDH (McIntyre et al., 1988). Western blots demonstrate that mAbs 3-10A, 4-4D and 36-4-1 are highly specific in detecting BDH among other mitochondrial proteins (Figure 1). Only mAb 16-7-1 (IgM-type) is not monospecific for BDH (Figure 1, lane 4). MAbs 3-10A and 4-4D detect rat liver BDH almost as well as bovine heart BDH (not shown). However, mAb 3-10A does not cross-react with human heart BDH on Western blots (not shown). MAb 36-4-1 reacts with rat liver BDH but not bovine heart BDH (Figure 1, lanes 9–11) or human liver BDH [in human liver mitochondria (20 μg) by Western blot, not shown]; the faint lower band for the rat liver SMV (Figure 1, lane 11) appears to be a proteolytie fragment of rat liver BDH, as it is also recognized by the anti-(bovine heart BDH) mAb 4-4D (not shown).

All IgG-type mAbs showed similar sensitivities for detecting apoBDH on dot-blots [< 10 ng of bovine heart BDH using a 1:100 dilution of each bovine heart BDH mAb (see Figure 2) and < 10 ng of rat liver BDH by mAb 36-4-1 at 1:160 dilution (approx 8 μg/ml) (not shown)]. Much larger amounts of the IgM-type mAb (16-7-1) (0.45 mg of γ-globulin/ml) were required to achieve weak detection of 10 ng of rat liver rat liver BDH on dot-blots (not shown). Under conditions similar to those shown in Figure 1, mAbs 2-12D and 3-2C (diluted 1:20) produce very weak reactions with BDH (0.25 μg) and no reaction with proteolytic fragments of BDH on Western blots (not shown). With both these mAbs, dot-blot assays show preferential detection of native BDH over BDH denatured on the blot before detection (Figures 2a and 2b). Although some reaction is detected with the denatured antigen (especially with higher amounts of applied antigen), the reaction is much stronger on blots that were not heated to denature the BDH. With mAbs 2-12D and 3-2C, both the Western blots and dot-blots are consistent with these mAbs recognizing specific 'confirmational' epitopes on native BDH. By comparison, mAb 4-4D probably recognizes a sequence epitope on BDH, since it shows similar detection of native and denatured BDH throughout most of the range tested (Figure 2d). The epitope for mAb 4-4D appears to be exposed in native BDH. The sequence-specific nature of the epitope for mAb 4-4D is confirmed by its reaction with a number of proteolytic fragments of BDH (see Figure 6). Although, for mAb 3-10A, the intensity of colour reaction for dot-blots with native and denatured BDH are not always comparable (Figure 2c), it is a sequence-specific mAb, since it binds to various proteolytic fragments of BDH on Western blots (see Figure 6) and its reaction with BDH is abolished by removing a few residues from the C-terminus of BDH (see Figure 3). Thus comparison of the reaction of a mAb with the native and denatured antigen on the same blot provides a rapid procedure for distinguishing mAbs with sequence-specific epitopes (e.g. mAb 4-4D) from those with conformational

Table 1 Characteristics of anti-BDH mAbs

The mAbs were prepared from mice immunized with either bovine heart or rat liver BDH as indicated. MAb 4-4D was from a mouse immunized with bovine heart BDH denatured by heating in SDS; the remaining five were from mice immunized with native BDH. Each mAb was tested for subtype (immunoglobulin type) by e.l.i.s.a. (Engvall, 1980). Cross-species reaction indicates whether each mAb produced against either bovine heart or rat liver BDH cross-reacts with rat liver or bovine heart respectively. Epitope specificity was determined by Western-blot analysis (Figure 1), dot-blot analysis (Figure 2) and, for the conformation-specific mAbs, by specific immunoprecipitation of BDH from detergent-solubilized SMV (not shown). Localization of sequence epitopes was by proteolytic cleavage (for C-terminal epitope 3-10A, see Figure 3; for 4-4D see Figures 6 and 7). Details of methodology are provided in the Experimental section.

mAb	Antigen	Immunoglobulin type	Species cross-reaction	Epitope diagnostic specificity
2-12D	Bovine heart BDH	IgG_1	Yes	Conformational
3-2C	Bovine heart BDH	IgG_1	Yes	Conformational (inhibiting)
3-10A	Bovine heart BDH	IgG_1	Yes	Sequence (C-terminal)
4-4D	Bovine heart BDH (denatured)	IgG_1	Yes	Sequence (central)
36-4-1	Rat liver BDH	IgG_1	No	Sequence
16-7-1	Rat liver BDH	IgM	Yes	Sequence

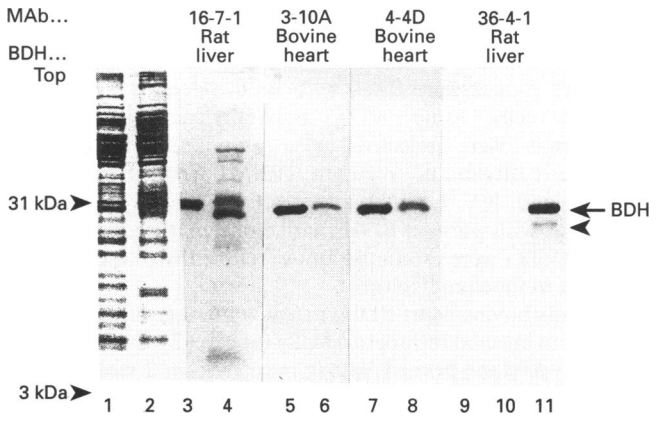

Figure 1 Specificity of mAbs for BDH on Western blots of SMV

Samples were subjected to SDS/PAGE [12.5% gel, 4% stack as described by Laemmli (1970)] and Western-blot analysis with mAbs as described in the Experimental section. Coomassie-stained samples of SMV (14 μg of protein each) are shown in lanes 1 (bovine heart) and 2 (rat liver). Purified bovine heart BDH (0.25 μg, as detected by Coomassie staining) migrates as a single band of approx 31.5 kDa (not shown). Western-blot samples: bovine heart BDH, 0.25 μg each, lanes 3, 5, 7, 9; bovine heart SMV, 5 μg each, lanes 4, 6, 8, 10; rat liver SMV, 10 μg, lane 11. mAbs used: 16-7-1, lanes 3 and 4; 3-10A, lanes 5 and 6; 4-4D, lanes 7 and 8; 36-4-1, lanes 9–11. The mAbs were prepared against either bovine heart or rat liver BDH as indicated (see Table 1). MAbs 3-10A and 4-4D were ascites fluids used at 1:100 dilution; 16-7-1 and 36-4-1 were chromatographically purified mAbs used at 0.084 mg/ml each.

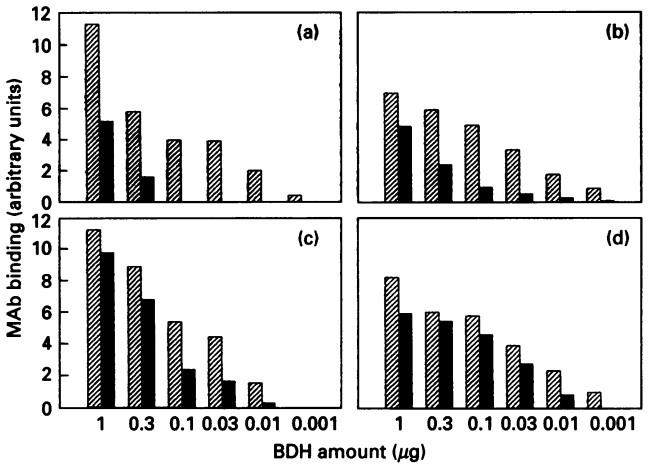

Figure 2 Reaction of mAbs with native and heat-denatured bovine heart BDH on dot-blots

Six different amounts of apoBDH were applied to duplicate nitrocellulose dot-blots, and one of the dot-blots was baked at 80 °C (under vaccuum) to heat-denature BDH and fix it to the nitrocellulose. Nitrocellulose membranes containing native apoBDH (hatched bars) and heat-denatured apoBDH (solid bars) were blotted with each mAb (1:100 dilution) as indicated. The height of each bar represents the photodensitometric intensity of each dot (arbitary units). (**a**), mAb 3-2C; (**b**) mAb 2-12D; (**c**) mAb 3-10A; (**d**) mAb 4-4D.

epitopes (e.g. mAb 3-2C), although with some mAbs (e.g. 2-12D and 3-10A), results are less discerning.

Of the four mAbs generated against bovine heart BDH, only mAb 3-2C inhibits the re-activation of bovine heart apoBDH almost entirely (> 90%) (Table 2). MAbs 3-10A and 2-12D give partial inhibition (approx 30%) of apoBDH. With MAb 3-2C, inhibition is almost complete when the antibody is incubated with apoBDH before reconstitution with activating phospholipid vesicles. However, even with excess antibody, only approx 50% inhibition is obtained when BDH is reconstituted with MPL vesicles before addition of mAb 3-2C. Furthermore, BDH–MPL complexes were inhibited approx 50% by mAb 3-2C when BDH activity was assayed with various substrate concentrations (near the K_m values). These results suggest that inhibition of BDH–MPL by mAb 3-2C does not significantly alter substrate K_m values, but decreases the limiting catalytic rate of the enzyme. For native BDH in SMV, no significant inhibition was observed even with mAb 3-2C, although polyclonal antibodies have been found previously to inhibit BDH in SMV (Churchill et al., 1983b; McIntyre et al., 1988). Although BDH in SMV was not significantly inhibited by mAb 3-2C, we found on the basis of immunoprecipitation studies that the epitope for this mAb is at least partially accessible. After incubating bovine heart SMV with either of the two conformational-specific mAbs (2-12D or 3-2C), BDH could be selectively immunoprecipitated from the membrane–mAb complexes solublized in 1% Triton X-100 (not shown). Thus the lack of inactivation of BDH in SMV by mAb 3-2C cannot simply be accounted for by masking of the epitope by the presence of other mitochondrial proteins.

Localization of the 3-10A epitope at the C-terminus of BDH

Western-blot analysis (Figure 3) shows that treatment of bovine heart apoBDH with carboxypeptidase Y abolishes the immunoreactivity of mAb 3-10A with BDH, whereas the immunoreactivities of mAbs 4-4D and 16-7-1 (not shown) are not affected,

Table 2 Study of inhibition of BDH activity with mAbs

Each mAb was incubated (1 h at 4 °C) with either purified BDH (bovine heart or rat liver) (0.1 mg/ml of either apoBDH or BDH–MPL complex) or SMV (bovine heart or rat liver, 1.0 mg of protein/ml) as antigen in a solution containing 10 mM sodium phosphate (pH 7.0), 5 mM NaCl and 0.5% BSA. The anti-(bovine heart BDH) and anti-(rat liver BDH) mAbs were tested against purified BDH from bovine heart and rat liver respectively. The anti-(bovine heart BDH) mAbs were ascites fluids at 50-fold final dilution. The chromatographically purified anti-(rat liver BDH) mAbs (36-4-1 and 16-7-1) were added to final concentrations of 83 μg/ml and 0.23 mg/ml respectively. Similar results were obtained with up to 5-fold higher concentrations of mAbs 3-2C or 4-4D. Samples of each of the antigen–mAb mixtures were assayed for BDH activity [37 °C, 5 mM NAD^+ and 20 mM (*R,S*)-hydroxybutyrate; see the Experimental section] which is expressed as the percentage of the appropriate control (samples diluted with buffer without mAb). The number of determinations is shown in parentheses; values with two or more determinations varied up to approx. 10% of the mean. Control BDH activities (μmol/min per mg of protein): 86 and 41 for purified bovine heart and rat liver BDH respectively, reconstituted with MPL, and 0.54 and 2.7 for bovine heart and rat liver SMV respectively. N.D., not determined.

MAb	BDH source	BDH activity (% of control)			
		ApoBDH	BDH–MPL	Bovine heart SMV	Rat liver SMV*
Control	–	100	100	100	100
2-12D	Bovine heart	72 (2)	93 (3)	101 (1)	96 (1)
3-2C	Bovine heart	7 (2)	50† (3) [51,52,49]‡	96 (2)	114 (1)
3-10A	Bovine heart	71 (2)	92 (3)	97 (1)	121 (1)
4-4D	Bovine heart	99 (2)	107 (3)	97 (1)	101 (1)
36-4-1	Rat liver	N.D.	109 (3)	N.D.	115 (2)
16-7-1	Rat liver	N.D.	103 (2)	N.D.	100 (2)

* The small apparent activation of BDH in rat liver SMV by three of the mAbs appears to be due to some instability of BDH activity in rat liver SMV under the conditions of incubation with the mAbs since control samples lost approx. 20% of activity. This apparent activation of BDH in rat liver SMV was abolished by addition of MPL, which stabilized BDH activity under the conditions of these experiments.

† Inhibition of activity of BDH–MPL by mAb 3-2C (at 1:50 dilution) was maximal within 15 min of the addition of the ascites fluid and remained constant thereafter for up to 2 h. Similar inhibition (approx. 50%) was obtained with dilutions of ascites fluid in the range from 1:50 to 1:10. With more dilute mAb (ascites 1:100 or 1:200), less inhibition was observed (approx. 60% and 70% residual activity respectively). Similar results were obtained with partially purified mAbs 3-2C and 4-4D, i.e. the IgG fraction prepared by 50%-satd. $(NH_4)_2SO_4$ precipitation from 3-2C ascites fluid gave approx. 50% inhibition of BDH–MPL, and mAb 4-4D was not inhibitory.

‡ With mAb 3-2C added to BDH–MPL, BDH activities shown in square brackets were measured using concentrations of substrates near the K_m values [K_m for NAD^+ approx. 0.1 mM and K_m for (*R*)-hydroxybutyrate approx. 1 mM (or 2 mM (*R,S*)-hydroxybutyrate) (Nielsen et al., 1973; Churchill et al., 1983a)]. The three values were obtained with the following NAD^+ and (*R,S*)-hydroxybutyrate concentrations, respectively: 0.1 mM and 20 mM; 5 mM and 2 mM; and 0.1 mM and 2 mM. With mAb 3-2C, similar inhibition was observed when BDH assays were carried out at 25 °C or when antibody was incubated with BDH–MPL at 37°. For samples of mAb 4-4D added to BDH–MPL, no significant inhibition was detected using any of the different assay conditions (different substrate concentrations or assay temperature).

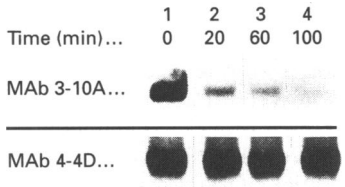

Figure 3 Time course of treatment of apoBDH with carboxypeptidase Y

ApoBDH (0.2 mg/ml) was incubated for the indicated times at 25 °C with 2% (w/w) carboxypeptidase Y as described in the Experimental section. Samples (0.25 μg of BDH/lane) were subjected to SDS/PAGE and Western blotting with a 1:100 dilution of mAb 3-10A or mAb 4-4D. Lane 1 shows untreated BDH. Samples were treated with carboxypeptidase Y for 20 min (lane 2), 60 min (lane 3), or 100 min (lane 4). Only the BDH band is shown for each lane since no other bands were detected by either mAb. The relative migration of BDH on the gel was not significantly altered by treatment with carboxypeptidase Y.

even after prolonged incubation with carboxypeptidase Y. Furthermore, since the 3-10A epitope is destroyed by C-terminal cleavage without a significant shift in the mobility of BDH on the gel, the 3-10A epitope must be located within a few residues of the C-terminus of BDH (less than 14 residues, estimated from the resolution of the SDS/PAGE under the conditions used, e.g. a proteolytic fragment of 29.5 kDa can be resolved from BDH; see Figure 6).

Effects of lipid environment on cleavage and inactivation of BDH by carboxypeptidase Y

Previous studies have shown that bovine heart or rat liver BDH, in the absence of lipids, or rat liver BDH when reconstituted with non-activating phospholipid vesicles lacking PC, are rapidly inactivated by treatment with carboxypeptidase. By contrast, BDH reconstituted with vesicles containing PC is resistant to inactivation by carboxypeptidases (Berrez et al., 1984; Maurer et al., 1985). Now, with the 3-10A epitope as a marker for the intact C-terminus, we have investigated whether PC-containing vesicles protect BDH from inactivation by preventing C-terminal cleavage or by stabilizing the active form of BDH even with C-terminal cleavage. The time courses of inactivation by carboxypeptidase are shown in Figure 4(a). It should be noted that BDH activity assays were carried out with a large excess of substrates, and that the initial activity in the absence of PC (BDH–PE/DPG vesicle complex) was less than 5% of the initial activity in the presence of PC (BDH–PC/PE/DPG complex). ApoBDH samples were reconstituted with PC/PE/DPG vesicles before enzymic activity was measured. Bovine heart BDH was protected from inactivation by carboxypeptidases only when reconstituted with vesicles containing PC. Western blots of carboxypeptidase-treated samples with mAb 3-10A (Figure 4b) show that reconstitution of BDH with PC-containing vesicles (PC/PE/DPG or PC alone) essentially prevented C-terminal cleavage, which was rapid in the absence of PC (apoBDH or BDH reconstituted with PE/DPG). Although the results shown in Figure 4(b) might suggest some loss of the 3-10A epitope for the activated BDH–PC/PE/DPG or BDH–PC complexes (lanes 3 and 4) compared with the control (lane 1), this effect was not reproducible and appears to be due to the non-linearity of the Western-blot colour reaction under the conditions of the experiment (for these studies, colour reaction times were prolonged in order to detect the small amount of 3-10A epitope in the cleaved samples in lanes 2 and 5). In other studies, we detected no significant time-dependent change in the amount of 3-10A epitope after treatment of BDH–PC/PE/DPG or BDH–PC with

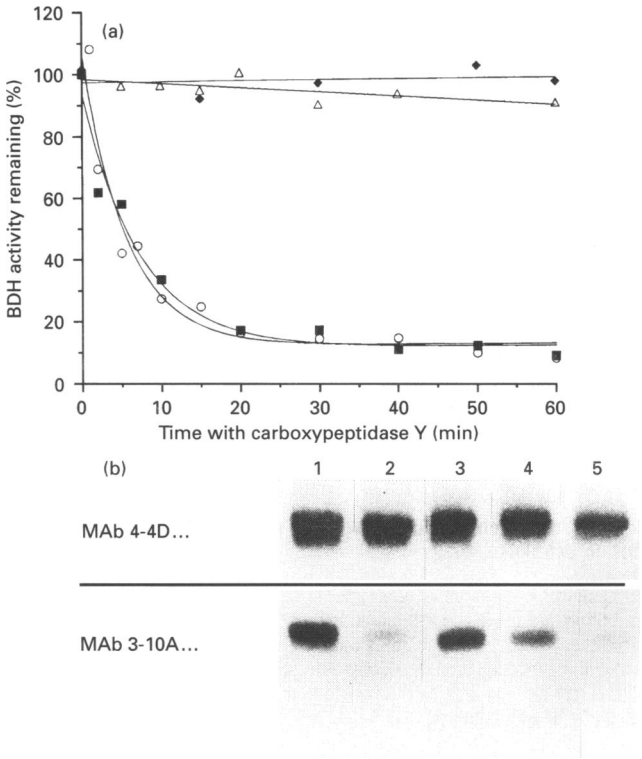

Figure 4 Cleavage and inactivation of bovine heart BDH by carboxypeptidase Y depends on its lipid environment

(**a**) Time course of inactivation. Samples of bovine heart apoBDH (○) or reconstituted complexes of BDH with phospholipid vesicles containing PC only (△), PC/PE/DPG (5:4:1, by phosphorus; ◆) or PE/DPG (9:1, by phosphorus; ■) were incubated with carboxypeptidase Y (2.5%, w/w) at 25 °C. Digestions were stopped at the indicated times by adding aprotinin (20 μg/ml, final concn.). ApoBDH samples were reconstituted with PC/PE/DPG vesicles in the cuvette (see the Experimental section) before being assayed for BDH activity. Enzyme activity of each sample was assayed with 10 mM NAD^+ and 200 mM (*R,S*)-3-hydroxybutyrate. The activity at each time point is presented as a percentage of the initial specific activity of that sample before incubation with carboxypeptidase Y. Initial specific activities (μmol of NADH/min per mg of BDH): re-activated apoBDH, 66.5 (37 °C); BDH–PC/PE/DPG, 28.8 (30 °C); BDH–PC, 20.1 (30 °C); BDH–PE/DPG, 1.34 (30 °C). (**b**) Detection of C-terminal cleavage of BDH by Western-blot analysis. Samples (0.25 μg of bovine heart BDH each) from 0 (lane 1) and 30 min digests with carboxypeptidase (lanes 2–5, apoBDH, BDH–PC/PE/DPG, BDH–PC and BDH–PE/DPG respectively) of the above time course were subjected to SDS/PAGE and Western blotting with mAbs 3-10A and 4-4D. Only the BDH bands are shown, since no other bands were detected by either mAb.

carboxypeptidase Y for up to 2 h (not shown). It can be seen (Figure 4) that C-terminal cleavage, sufficient to destroy the 3-10A epitope and inactivate BDH, did not cause a significant change in the molecular mass of BDH (Figure 4b, lanes 2 and 5), indicating that removal of only a few C-terminal residues was sufficient to inactivate BDH and eliminate the 3-10A epitope. These results confirm that only reconstitution of BDH with phospholipid vesicles containing PC protects the enzyme from inactivation by carboxypeptidases and show that this protection is due to the inaccessibility of the C-terminus of the active form of the enzyme in phospholipid bilayers containing PC.

Both bovine heart and rat liver apoBDH can be re-activated by soluble short-chain PC in the absence of a micelle or phospholipid bilayer (Gazzotti et al., 1975; Cortese et al., 1982). We examined whether activation of BDH by monomeric $PC(8:0)_2$ would protect BDH from carboxypeptidase as does activation by bilayers containing PC. Bovine heart apoBDH was treated with carboxypeptidase in the absence of $PC(8:0)_2$ or in the presence of 135 μM monomeric $PC(8:0)_2$, which re-activates BDH to 80% or more of the optimal specific activity obtained

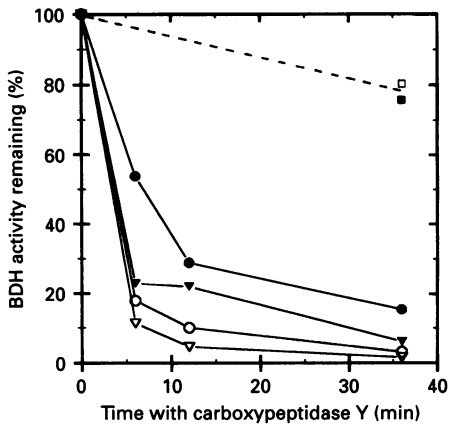

Figure 5 Carboxypeptidase Y inactivation of bovine heart apoBDH in the absence and presence of an activating concentration of monomeric PC (8:0)$_2$

Bovine heart-apoBDH was incubated at 0.1 mg/ml, at approx. 23 °C, in a solution containing 50 mM Hepes, 1 mM EDTA, 1 mM DTT, 5% (v/v) ethanol, pH 7.4, in the absence (■, ●, ▼) or presence (□, ○, ▽) of 135 μM PC(8:0)$_2$. Carboxypeptidase was added to 2% (w/w) BDH and cleavage was quenched at the indicated times by adding aprotinin (8-fold molar excess over carboxypeptidase Y) and phenyl methanesulphonylfluoride (1 mM final). Portions of quenched samples were re-activated with PC/PE/DPG vesicles (○, ●) or 135 μM monomeric PC(8:0)$_2$ (▽, ▼) for 15 min at 30 °C in the presence of 10 mM NAD^+, and then assayed for activity at 30 °C by adding (*R*)-3-hydroxybutyrate to 10 mM (○, ●) or 20 mM (▽, ▼). Activities are presented as percentage of the activity before addition of carboxypeptidase Y. Initial specific activities (μmol/min per mg of BDH): 59 (○); 46 (●); 48 (▽); 33 (▼). The uncleaved controls (□, ■) lost 20–25% of the initial activities after 36 min.

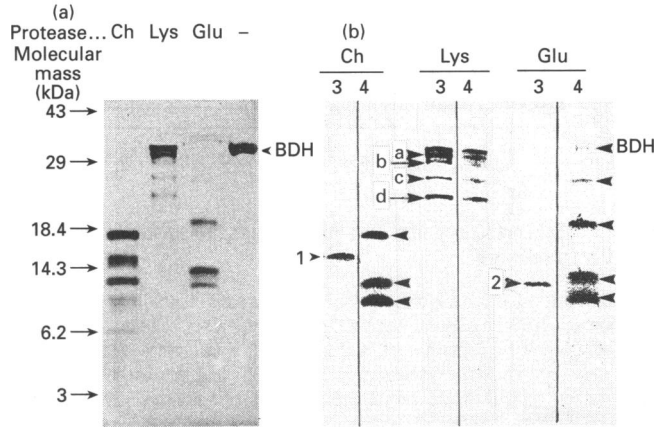

Figure 6 Partial cleavage of BDH by three different endopeptidases

Bovine heart BDH (0.12 mg/ml) was partially unfolded with SDS as described in the Experimental section and then incubated with 20% (w/w) α-chymotrypsin (Ch), 5% (w/w) lysine-specific endopeptidase (Lys) for 25 min or 0.8% (w/w) glutamate-specific endopeptidase (Glu) for 10 min. SDS/PAGE was carried out using a 12% gel with 4% stack (Schägger and Von Jagow, 1987). (**a**) Silver-stained gel of the digests (0.3 μg of BDH/lane). (**b**) Western blots (0.18 μg of BDH/lane) comparing the fragments detected by mAbs 3-10A and 4-4D (labelled 3 and 4 respectively) for each specific digest. Photodensitometry of the silver-stained gel and Western blot were used to estimate the molecular-mass values for fragments by comparison with R_F values for the different molecular-mass standards (see the Experimental section), the positions of which are indicated at the left side of (**a**). The sizes (kDa) of the fragments (denoted by arrowheads in **b**) which react with mAb 3-10A or 4-4D or both (as noted in parentheses by 3, 4 or 3/4) are as follows: α-chymotryptic fragments: 16.9 (4), 14.6 (3) (fragment no. 1), 10.7 (4) and 8.5 (4); endo-Lys fragments denoted a, b, c and d: 29.5 (3/4), 28.2 (3/4), 25.2 (3/4) and 22.5 (3/4); and endo-Glu fragments: 24.6 (4), 18.5 (4), 11.3 (4), 10.1 (3) (fragment no. 2) and 8.8 (4). Arrowhead 1 marks the largest fragment (14.6 kDa) detected by mAb 3-10A which was not detected by mAb 4-4D, and arrow d marks the smallest fragment (22.5 kDa) detected by both mAbs; these establish the boundaries of the region containing the 4-4D epitope (see Figure 7).

Table 3 Retention of conformational epitope (3-2C) after C-terminal cleavage of BDH

ApoBDH [without PC(8:0)$_2$], was incubated without (control) or with 1% (w/w) carboxypeptidase, under conditions described for Figure 5. After a 40 min incubation, proteolysis was stopped by addition of protease inhibitors (see Figure 5). Samples were taken for measurement of BDH activity and dot-blot analysis. For assays of BDH activity [30 °C, 10 mM NAD^+ and 20 mM (*R,S*)-3-hydroxybutyrate], samples were re-activated with PC/PE/DPG vesicles (see the Experimental section). For dot-blot analysis, samples were diluted 50-fold with TBS buffer containing 0.2 μg of aprotinin/ml and 100 μl portions (0.2 μg of BDH each) were applied to nitrocellulose (approx. 32 mm^2 dots). Duplicate dot-blot samples were then detected with mAb 3-10A or 3-2C and analysed by photodensitometry, as described in the Experimental section. The dot-blot intensities are given in arbitrary units (detection of BDH is not strictly linear over the range of intensities shown here; see Figure 2).

ApoBDH	BDH activity (μmol/min per mg)	Detection by mAb 3-10A	Detection by mAb 3-2C
Control	25	3910	3990
Carboxypeptidase-treated	4	920	3620

when it is reconstituted with vesicles containing PC (T. M. Duncan, unpublished work). As shown in Figure 5, monomeric PC(8:0)$_2$ did not protect bovine heart BDH from inactivation by carboxypeptidase. Results were similar whether BDH activity was assayed directly with PC(8:0)$_2$ or after incubation with excess vesicles of PC/PE/DPG (Figure 5, compare open with closed symbols). Both BDH samples [with or without 135 μM PC(8:0)$_2$] were inactivated by more than 80% after 36 min with 2% carboxypeptidase (Figure 5). Since the controls for these studies lost 20–25% activity, additional carboxypeptidase digestion studies were carried out with BDH in solution (aqueous BDH) under a variety of conditions that were found (T. M. Duncan and J. O. McIntyre, unpublished work) to stabilize the enzyme in the absence of a phospholipid bilayer, including in the presence of either PC(8:0)$_2$ at concentrations above the critical micellar concentration or other soluble amphiphiles, such as 5 mM Zwittergent 3-08 (a zwitterionic detergent with a critical micellar concentration higher than 100 mM). For aqueous BDH in the presence of 135 μM monomeric PC(8:0)$_2$, 300 μM PC(8:0)$_2$ (micellar) or 5 mM Zwittergent 3-08 (monomeric detergent), 40 min of treatment with 1% (w/w) carboxypeptidase produced comparable and almost complete inactivation (< 20% remaining activity; not shown); in fact, rates of inactivation were somewhat faster in the presence of either 135 or 300 μM PC(8:0)$_2$ ($t_{\frac{1}{2}}$ < 10 min) than in the absence of PC(8:0)$_2$ ($t_{\frac{1}{2}}$ ~ 15 min). Thus inactivation of apoBDH by carboxypeptidase was not prevented by monomeric or micellar PC(8:0)$_2$ or by monomeric detergent. It is noteworthy that carboxypeptidase-digested bovine heart apoBDH could not be re-activated by either bilayer or soluble PC (Figure 5, closed circles and triangles respectively), indicating that the inactivation is not simply related to loss of the ability of the cleaved apoBDH to insert into the bilayer. In addition, as shown in Table 3, detection of BDH by conformation-specific mAb 3-2C was essentially unaffected by C-terminal cleavage of BDH sufficient to inactivate the enzyme by more than 80%. Similar results (not shown) were obtained when BDH was cleaved by carboxypeptidase in the presence of either monomeric

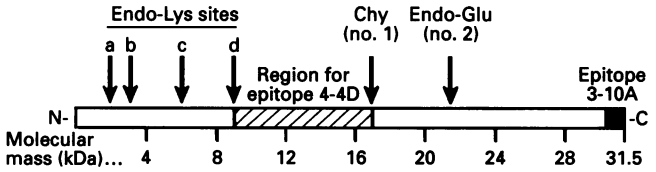

Figure 7 Schematic map of proteolytic sites and two sequence epitopes on the BDH polypeptide

A molecular mass of 31.5 kDa is assumed for BDH on the basis of SDS/PAGE analysis (Bock and Fleischer, 1975), and distances from the N-terminus (left) are noted below the polypeptide. The filled region at the C-terminus indicates the location of the epitope for mAb 3-10A as determined in Figure 3. Molecular-mass values for proteolytic fragments are given in Figure 6. Arrows indicate approximate positions of proteolytic sites for lysine-specific protease (endo-Lys), α-chymotrypsin (Chy) and glutamate-specific protease (endo-Glu). The region containing the 4-4D epitope between 9.0 and 16.9 kDa from the N-terminus (hatched) is defined at the left and right by fragments d and no. 1 respectively, shown in Figure 6, i.e. between 22.5 and 14.6 kDa from the C-terminus of BDH.

(135 μM) or micellar (300 μM) $PC(8:0)_2$ or with monomeric Zwittergent 3-08 (5 mM). Since mAb 3-2C shows preferential detection of the 'native' conformation of apoBDH (Figure 2), these results indicate that C-terminal cleavage does not simply cause apoBDH to unfold. Therefore the combined results of Figures 4, 5 and Table 3 indicate that protection of the C-terminus of BDH from carboxypeptidase requires that BDH be membrane-bound and that PC be present in the bilayer.

Proteolytic mapping of BDH

To determine the relative location of BDH sequence epitopes, partial proteolytic digests of BDH were compared on Western blots with each mAb. With the 3-10A epitope as a C-terminal marker on BDH, other proteolytic sites and sequence epitopes have been mapped relative to the C-terminus. Comparison of bovine heart BDH fragments recognized by mAbs 3-10A and 4-4D (Figure 6) shows several fragments containing both epitopes and other distinct fragments that contain only one of those epitopes. On the basis of these results, the 4-4D epitope is located at least 14.6 kDa but no further than 22.5 kDa from the C-terminus of BDH [as defined by the largest peptides that react with 3-10A only (arrowhead no. 1) and the smallest peptide (arrowhead d) that reacts with both 3-10A and 4-4D (Figure 6b)]. Thus the 4-4D epitope is located within a region that represents about 25% (7.9 kDa) of the BDH polypeptide (i.e. between 9 and 16.9 kDa from the N-terminus). MAb 16-7-1 detects a subset of the fragments recognized by mAb 4-4D (not shown), indicating that these two mAbs recognize epitopes that are near each other on the BDH sequence. The approximate locations of proteolytic sites and the 4-4D epitope on BDH, relative to the C-terminal 3-10A epitope, are shown schematically in Figure 7. With rat liver BDH, partial cleavage with any of the three proteases yields fragmentation patterns (not shown) similar to those for bovine heart BDH (Figure 6a). Further, the sequence-specific and cross-reacting mAbs (4-4D, 3-10A and 16-7-1) detect specific fragments of rat liver BDH similar to those for bovine heart BDH (Figure 6b) (not shown) consistent with previous data (McIntyre et al., 1988; Marks et al., 1992; Churchill et al., 1992), indicative of cross-species conservation of this enzyme.

DISCUSSION

We have prepared mAbs against BDH and demonstrated their utility for studying structure–function relationships in this lipid-requiring membrane-bound enzyme. Five of six characterized mAbs, two of which have conformational epitopes, were found to cross-react with both bovine heart and rat liver BDH, confirming the evolutionary conservation of this mitochondrial enzyme. Only one of the six mAbs (3-2C), which reacts with a conformational epitope, was found to give marked inhibition of BDH activity. Proteolytic cleavage and mapping studies show that one of the sequence-specific epitopes (3-10A) is at the C-terminus of the enzyme. The key findings from our studies are that the C-terminus is important for the function of BDH and that PC modulates the orientation of BDH in the bilayer. The C-terminus of BDH is protected from cleavage and inactivation by carboxypeptidase only when BDH is membrane-bound and PC is present in the bilayer. Our results are consistent with the interpretation that the PC-binding site is at the C-terminus of the molecule.

The C-terminus is clearly important for the function of BDH. Cleavage of a relatively few amino acids (14 or fewer) from the C-terminus of apoBDH with carboxypeptidase removes the epitope for mAb 3-10A (C-terminal epitope) and is sufficient to inactivate the enzyme. The loss of the ability to re-activate carboxypeptidase-treated apoBDH is not simply related to lack of proper insertion into the bilayer since the truncated enzyme was not activated by either bilayer or soluble PC (Figure 5) even though it retained its conformational epitope recognized by mAb 3-2C (Table 3). For BDH reconstituted into phospholipid bilayers containing PC, both the C-terminal epitope and the activity of the enzyme were protected from cleavage and inactivation by carboxypeptidase (Figure 4). In contrast, BDH activated by aqueous monomeric concentrations of short-chain PC in the absence of any bilayer is rapidly cleaved and inactivated by carboxypeptidase (Figure 5). The difference in accessibility of the C-terminus of BDH activated by bilayer versus soluble PC suggests that, in bilayers containing PC, the C-terminus is masked from the carboxypeptidase. A possible explanation is that activation of membrane-bound BDH by PC induces a conformational change in which the C-terminus moves from an aqueous surface of the enzyme to within the hydrophobic interior of the bilayer, thereby making the C-terminus inaccessible to carboxypeptidase. Activation by soluble monomeric PC may induce a similar conformational change in BDH but, in the absence of a bulk hydrophobic phase, the C-terminus remains exposed to carboxypeptidase. Alternative explanations include the possibility that the difference between bilayer and aqueous BDH is related to the difference in the equilibrium binding of BDH to short-chain PC versus bilayers containing PC. BDH reconstituted into membranes or in phospholipid vesicles containing PC cannot readily be dissociated from the bilayer (Gazzotti et al., 1975). By contrast, although BDH can be almost maximally activated by short-chain PC, the binding of soluble lipids to BDH is relatively weak, e.g. the BDH–$PC(8:0)_2$ complex can readily be dissociated by gel-exclusion chromatography (Gazzotti et al., 1975) or dilution (J. O. McIntyre, unpublished work). Thus it remains possible that $PC(8:0)_2$ also masks the C-terminus of BDH without significantly altering the rate of cleavage by carboxypeptidase which may displace the weakly bound $PC(8:0)_2$ from BDH. For BDH reconstituted into phospholipid vesicles, the interpretation is simpler and profound. The presence of PC in the bilayer results in the protection of the C-terminus from carboxypeptidase cleavage and consequent inactivation of the enzyme. Thus the orientation of BDH in the membrane is modulated by the presence of PC in the bilayer and appears to reflect a conformational transformation in BDH which can be attributed to the allosteric activation of this lipid-requiring enzyme by PC. These results are in accord with the

interpretation that the C-terminal domain of BDH contributes to the PC-binding site as predicted from analysis of the primary sequence as compared with other short-chain alcohol dehydrogenases (Marks et al., 1992).

The availability of a probe for the C-terminus of BDH (mAb 3-10A) enabled studies to begin to map proteolytic cleavage sites on BDH. Such studies locate the 4-4D epitope in a region between 9 and 16.9 kDa from the N-terminus (Figure 7). The specific cleavage sites for the various proteases (see Figures 6 and 7) remain to be defined. It is now feasible to achieve a detailed cleavage map of BDH including the location of sequence epitopes. MAbs that recognize different portions of BDH sequence should be valuable in mapping the topology of membrane-bound BDH, and we have begun studies of this type (Duncan et al., 1989; Adami et al., 1990).

Only one (mAb 3-2C) of the six mAbs studied here gave marked inhibition of BDH activity. Further, this conformation-specific mAb had its greatest inhibitory effect when incubated with apoBDH before reconstitution of the enzyme with vesicles containing PC. This suggests that the epitope recognized by mAb 3-2C may be in a region of BDH involved in membrane binding or that, on activation by PC, the epitope may undergo a conformational change which weakens its interaction with mAb 3-2C. Localizing residues of BDH involved in this conformational epitope may help identify the lipid- or PC-binding region of BDH. The lack of any significant effect of mAb 3-2C on the apparent K_m of BDH for nucleotide or substrate suggests that the epitope is not in the catalytic domain of the enzyme. The small inhibition of apoBDH by mAbs 2-12D and 3-10A without any significant effect on the activity of BDH reconstituted into bilayers (BDH–MPL) (Table 2) suggests that these epitopes are accessible for BDH in solution but masked in membrane-bound BDH. For 3-10A (C-terminal epitope), the inhibition data are consistent with the carboxypeptidase-cleavage results which show that the C-terminus of BDH is protected after reconstitution into bilayers containing PC. Although the six mAbs that we have prepared do not appear to have epitopes in the active centre of the enzyme, clearly antigenic epitopes are present and remain to be identified since both rat liver and bovine heart BDH are strongly inhibited by polyclonal antibodies (Churchill et al., 1983b; McIntyre et al., 1988).

The cross-reactivity of five of the six mAbs between the bovine heart and rat liver enzymes (including the two conformational epitopes) confirms the evolutionary conservation of this enzyme, as already noted by McIntyre et al. (1988). However, the specificity of mAb 36-4-1 for rat liver BDH reinforces the fact that they are not identical. These results are consistent with recently published amino acid sequence data for BDH which show extensive sequence identity between human and bovine heart BDH (89% identical residues) (Marks et al., 1992) and with rat liver BDH (Churchill et al., 1992) (86% identical residues in human heart and rat liver BDH). The C-terminal epitope for mAb 3-10A is conserved in bovine heart and rat liver BDH but is not detected in human heart BDH, although polyclonal antibodies show cross-reactivity between bovine and human heart BDH. The cross-reactivity pattern with mAb 3-10A for the C-terminal epitope is consistent with the amino acid sequence data for the C-terminal region of the enzyme from the three species; the 13-amino acid C-terminal peptide sequence of the bovine heart enzyme [corresponding to residues 284–297 of both human heart and rat liver BDH (Marks et al., 1992; Churchill et al., 1992)] matches the corresponding rat liver sequence with one conservative substitution (Arg replacing Lys-293 of the rat liver enzyme), whereas the human heart enzyme has three substitutions [Phe, Arg and His in the bovine enzyme replacing Leu-286, Met-293 and Arg-297 (C-terminal) of human heart BDH]. Since this region of BDH is important for the activation by PC, it will be of interest to compare the interaction of PC with the human and bovine heart and rat liver forms of the enzyme.

The importance of the C-terminus for activation of another lipid-requiring enzyme, *Escherichia coli* pyruvate oxidase, has been reported (Grabau et al., 1989): directed mutagenesis suggested that disruption of a putative α-helix close to the C-terminus destroyed the ability of the enzyme to bind lipids. More recent studies in our laboratory indicate that the C-terminus of BDH is also important for binding the enzyme to membranes (T. M. Duncan, P. Adami, N. Latruffe, M.-F. Sun, S. Fleischer and J. O. McIntyre, unpublished work). The mAbs characterized here should prove valuable in further studies of BDH membrane topology and its relationship to the activation of BDH by PC.

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