Wild Type and Mutant Human Heart (*R*)-3-Hydroxybutyrate Dehydrogenase Expressed in Insect Cells[†]

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ABSTRACT: (*R*)-3-Hydroxybutyrate dehydrogenase (BDH) is a lipid-requiring mitochondrial enzyme with a specific requirement of phosphatidylcholine (PC) for function. PC is an allosteric activator that enhances NAD(H) binding to BDH. The enzyme serves as a paradigm to study specific lipid−protein interactions in membranes. Analysis of the primary sequence of BDH, as determined by molecular cloning, predicts that lipid binding and substrate specificity are contributed by the C-terminal third of the protein [Marks, A. R., McIntyre, J. O., Duncan, T. M., Erdjument-Bromage, H., Tempst, P., & Fleischer, S. (1992) *J. Biol. Chem. 267*, 15459−15463]. The mature form of human heart BDH has now been expressed in catalytically active form in insect cells (Sf 9, *Spodoptera frugiperda*) transfected with BDH-cDNA in baculovirus. Endogenous PC in the insect cells fulfills the lipid requirement for the expressed BDH since enzymatic activity is lost upon digestion with phospholipase A_2 and restored selectively by reconstitution with PC vesicles. The K_ms for NAD^+ and (*R*)-3-hydroxybutyrate (R-HOB) of expressed BDH are similar to those for bovine heart or rat liver BDH in mitochondria. Replacing Cys242 (the only cysteine in the C-terminal domain) with serine by site-directed mutagenesis resulted in a 10-fold increase in K_m for R-HOB with no change in the K_m for NAD^+, indicating a role for Cys242 in substrate binding. Carboxypeptidase cleavage studies had indicated a requirement of the C-terminal for catalysis and a role in lipid binding [Adami, P., Duncan, T. M., McIntyre, J. O., Carter, C. E., Fu, C., Melin, M., Latruffe, N., & Fleischer, S. (1993) *Biochem J. 292*, 863−872]. We now show that deletion of twelve C-terminal amino acids to form a truncated BDH mutant results in loss of enzymic function. The expression in Sf 9 cells of the constitutively active full-length mature form of human heart BDH and the first expression and characterization of BDH mutants validate this system for structure−function studies of BDH.

(*R*)-3-Hydroxybutyrate dehydrogenase (BDH;[1] EC 1.1.1.30) is a lipid-dependent mitochondrial enzyme with an absolute and specific requirement of phosphatidylcholine (PC) for function (Fleischer et al., 1966; Berrez et al., 1984).[2] The purified enzyme has served as a paradigm to study the role of lipid and the nature of lipid−protein interactions in membranes (Berrez et al., 1984; Fleischer & McIntyre, 1985; Sandermann et al., 1993). The enzyme is tetrameric (McIntyre et al., 1983) and present on the matrix face of the inner mitochondrial membrane (McIntyre et al., 1978). The mature enzyme of 297 amino acid residues is formed after cleavage of the N-terminal leader peptide (Milhara et al., 1982; Marks et al., 1992). Although BDH behaves as an integral membrane protein, it exhibits an amphipathic mode of interaction with the lipid bilayer (McIntyre et al., 1978, 1979). PC serves as an allosteric activator of BDH in that it enhances NAD(H) binding to BDH (Gazzotti et al., 1974; Rudy et al., 1989).

The recent molecular cloning and sequencing of BDH from human heart (Marks et al., 1992) and rat liver (Churchill et al., 1992) define it as a member of the short-chain (non-metalloenzyme) alcohol dehydrogenase (SC-ADH) family of proteins. This family is composed of over 20 members which share on average approximately 25% homology at the amino acid level (Persson et al., 1991). The N-terminal two-thirds of BDH shares features with the SC-ADH family of proteins, including three Gly residues (Gly16, Gly20, Gly22) which are likely to be important in dinucleotide binding (Jornvall et al., 1984; Wierenga et al., 1985; Ghosh et al., 1991) and three strictly conserved polar amino acids (Ser149, Tyr162, Lys166) which have been postulated to play a role in catalysis (Persson et al., 1991). The C-terminal region of BDH is largely unique and therefore likely confers to the protein both the substrate specificity and the phospholipid binding/activation properties (Marks et al., 1992).

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[1] Abbreviations: BDH, (*R*)-3-hydroxybutyrate dehydrogenase; EDTA, ethylenediaminetetraacetic acid; HOB, hydroxybutyrate; BH, bovine heart; DPG, diphosphatidylglycerol; HH, human heart; K_m^{HOB} and K_m^{NAD+}, apparent Michaelis constants for R-HOB and NAD^+, respectively; mBDH, mature form of BDH; PC, phosphatidylcholine; PCR, polymerase chain reaction; PE, phosphatidylethanolamine; PL, phospholipid; SC-ADH, short-chain alcohol dehydrogenases (Persson et al., 1991); SMV, submitochondrial vesicles.

[2] This work is dedicated to the memory of Dr. Becca Patras Fleischer (deceased January 28th, 1994) who carried out pioneering studies of β-hydroxybutyrate dehydrogenase and was a devoted scholar of biomembranes.

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We report here the use of the baculovirus protein expression system to express catalytically active BDH in Sf 9 insect cells. While a number of mitochondrial enzymes have been expressed in insect cells (Lithgow et al., 1991; Takagi et al., 1992; Tamura et al., 1992), catalytic activity has not been observed in all cases, and, at least for one mammalian protein, novel cleavage of the mitochondrial leader sequence was found in the insect cells (Lithgow et al., 1991). In our studies, the mitochondrial targeting sequence was deleted from precursor BDH-cDNA yielding high-level expression of the mature form of the enzyme in the membrane fraction of Sf 9 cells. Expressed BDH exhibits typical dependence on PC for catalysis. Studies with mutated variants of BDH expressed in this system confirm and extend prior observations regarding the importance of Cys242 and the C-terminal domain of BDH for enzymic function. The studies presented here describe the first mutants of BDH and indicate that this expression system can be used to investigate the molecular cytology of this lipid-requiring enzyme.

EXPERIMENTAL PROCEDURES

Oligonucleotide PCR Primers. CCGGTACCTAAATATGGCCAGTGCGGCGGAGCCG (primer 1), CGGCCCAGCATGCTGCTG (primer 2), GGAGGCCTTCAGTGGGTCATGATCTGCAT (primer 3), CCCCATGGACTACTACTGG (primer 4), CCCCATGGACTACTACCAGTGGCTGCGA (primer 5), TTAGAGGCCTCTGCCTGCC (primer 6), GCCACTGCTGCTGTAGGTCTC (primer 7).

DNA Sequencing Primers. GCAGTACGGGGAGCGGGC (primer A), GAGACCTACTGCAGCAGT (primer B), CCGGATTATTCATACCG (primer C), GTGGGAGGAGCTGCCT (primer D).

PCR Reactions. PCR was performed in a thermal cycler (Perkin-Elmer-Cetus Corp., Norwalk, CT) in 100 μL reactions containing 0.5 μM of each primer, 1 ng of template DNA, 200 μM of each dNTP, 1.25 or 2.50 mM $MgCl_2$, 10 μL of 10× PCR buffer (Promega, Madison WI), and 4 units of Taq DNA polymerase (Promega). The reactions were amplified in 35 cycles consisting of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C, followed by 10 min at 74 °C.

DNA Constructions. The full-length cDNA encoding human heart BDH (Marks et al., 1992) in pBlueScript SK(−) vector was used as template DNA in a PCR reaction to both remove the mitochondrial targeting sequence and add an ATG initiation site so as to obtain cDNA encoding only the mature form of BDH (mBDH-cDNA). Primer 1 (see above) creates a *Kpn*I site 5′ to a Kozak sequence (TAAAT) and ATG site. Using primers 1 and 2, a 468 bp *Kpn*I-*Sph*I fragment was produced by PCR and then double-digested with *Kpn*I and *Sph*I. This fragment was purified and ligated to the BDH-BlueScript vector. The resultant mBDH-BlueScript construct encodes the mature BDH polypeptide (Mihara et al., 1982; Marks et al., 1992) with methionine added at the N-terminus. To directionally clone the mBDH-cDNA into the baculovirus transfer vector, pVL1393, the mBDH-BlueScript vector was digested with *Kpn*I, blunt-ended with Klenow fragment, and digested with *Eco*RI. The transfer vector, pVL1393 (provided by Dr. R. Magnusson, Mount Sinai School of Medicine, New York) was digested with *Sma*I, gel purified, and redigested with *Eco*RI. The 1.4 kb *Eco*RI blunt-end fragment of mBDH-cDNA was then ligated into pVL1393. The mBDH-cDNA constructs in pVL1393 were confirmed by DNA sequencing. Two constructs of mBDH-cDNA (A1-1 and B1-2), products of separate PCR reactions, were found to each contain unintended PCR-induced nucleotide errors which in each case resulted in single amino acid substitutions; for B1-2, a valine is substituted for Met92, and for A1-1 a threonine is substituted for Ser24 (positions refer to those for mature BDH) (Marks et al., 1992). To reconstruct wild-type BDH, the *Eco*NI fragment of the original full-length BDH-cDNA in BlueScript was exchanged for the *Eco*NI fragment contained in the B1-2 mBDH baculovirus vector. These fragments were ligated, and the correct orientation was screened by *Pst*I digestion. The sequence of wild type BDH-cDNA in baculovirus was then confirmed by DNA sequence analysis.

The DNA sequence of both mBDH-cDNA and the original full-length cDNA in pBlueScript SK(−) revealed two errors between residues 370 and 403 in the previously reported nucleotide sequence of HH-BDH (Marks et al., 1992). The correct nucleotide sequence (residues 370−403) is GTG GAG ATT GTC CGC TCG AGC CTG AAG GAC CCT, yielding an amino acid sequence of VEIVRSSLKDP (residues 78−88 of mBDH). With this corrected sequence there is 88% identity between the mature forms of human heart and rat liver BDH (Figure 1).

A COOH-terminal twelve amino acid deletion clone of mBDH (CTT-D12) was produced by first generating a 57 bp fragment with primers 5 and 6 using BDH B1-2 vector DNA as template in a PCR reaction. This fragment was digested with *Nco*I and *Stu*I and was ligated into the wild-type mBDH-cDNA in the baculovirus vector.

The substitution of Cys for Ser at position 242 (Cys242→Ser) was produced by site-directed mutagenesis (Muta-Gene Phagemid *in vitro* mutagenesis kit version 2, Bio-Rad Corp., Hercules, CA) based on the negative selection of uracil-containing template DNA, as initially described by Kunkel (1985). The B1-2 mBDH in pBlueScript was used to prepare single-stranded uracil-containing template DNA; primer 7 was phosphorylated by polynucleotide kinase, annealed to template at 70 °C, and allowed to cool to 30 °C over a 40 min period, followed by synthesis of covalently closed circular DNA as described in Bio-Rad protocols using T4 DNA ligase and T7 DNA polymerase in a 90 min reaction at 37 °C. Recombinant clones were screened for by the resultant deletion of a *Pst*I site. The Val92 PCR-induced mutation found to be present in B1-2 BDH (see above) was then corrected by first preparing a 190 bp *Ppu*MI-*Sph*I fragment from the full-length BDH-cDNA clone in pBlueScript. The corresponding insert was removed from the Ser242 BDH, and the 190 bp fragment containing the wild-type Met92 was ligated in its place. To clone BDH Ser242 cDNA into baculovirus transfer vector, an *Eco*NI fragment was derived from the Ser242 BDH and ligated into the wild-type BDH baculovirus transfer vector DNA which had been *Eco*NI digested, treated with calf intestinal alkaline phosphatase, and gel purified. A construct with correct orientation was confirmed by DNA sequence analysis.

Cell Culture and Transfection. Sf 9 cells (a clonal line derived from the ovary of *Spodoptera frugiperda*) were a gift of Dr. R. Magnusson and were grown at 27 °C in attached form using either 25 cm^2 or 75 cm^2 canted neck flasks [Falcon T25 or T75 flasks, see Miller (1988) and O'Reilly et al. (1994)] with TNM-FH medium [Grace's medium (Gibco, Gaithersburgh, MD) with Yeastolate and

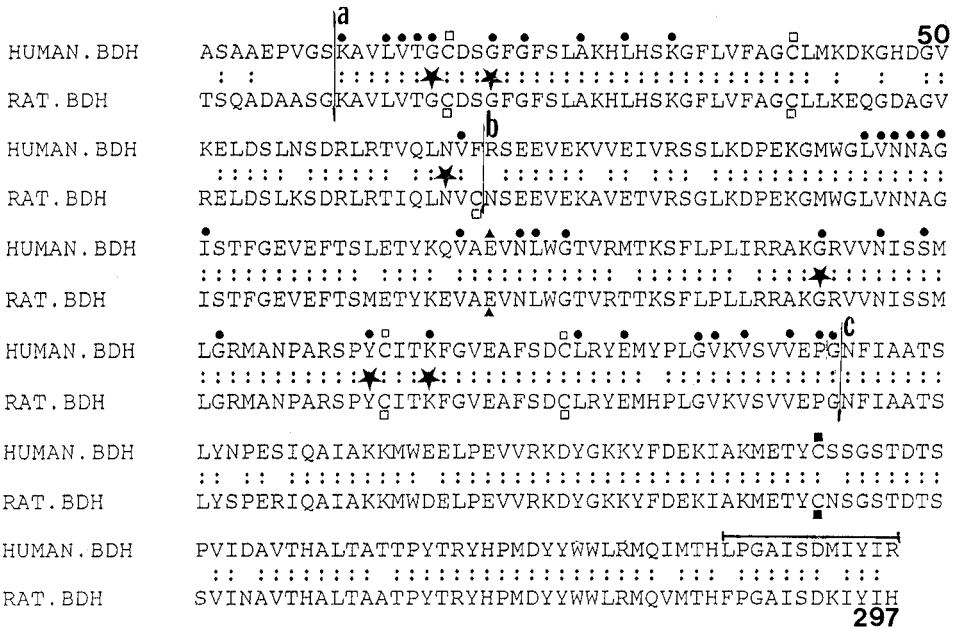

FIGURE 1: Amino acid sequences and homology of the mature forms of human heart and rat liver BDH. The predicted amino acid sequences of the enzyme from the two species are from cDNA cloning studies (Marks et al., 1992; Churchill et al., 1992) with the leader peptides omitted. Residues 79−87 of HH-BDH have been changed on the basis of a corrected BDH-cDNA sequence (see Experimental Procedures). Of the 297 residues in each protein, 262 are identical as indicated by "colons" between the aligned sequences (88% identity). Specific residues are marked as follows: ●, residues identical with conserved residues of SC-ADH; ★, locations of residues conserved in twenty SC-ADH; □, cysteine residues; ■, Cys242; ▲, Glu120. Letters a−c denote the first residue in each of the three putative domains of BDH, i.e., the NAD(H) site, substrate/catalytic site, and lipid/PC binding domains, respectively. The bar indicates the twelve residues deleted from HH-BDH in the CTT-D12 variant.

lactalbumin hydrolysate (Gibco) both added to 3.3 g/L] containing 10% fetal calf serum. Antibiotics were routinely added consisting of Fungizone 2.5 μg/mL and gentamycin sulfate at 50 μg/mL. Linearized, modified baculovirus DNA was obtained from PharMingen (BaculoGold Transfection Kit, PharMingen, San Diego, CA) and was transfected along with the constructs in pVL1393 as per PharMingen protocols.

Purification of Recombinant Baculovirus Clones. Transfection medium was amplified either once or twice in Sf 9 cells (in either T25 or T75 flasks) each over 4−5 days. Cells were either directly harvested or in some cases the recombinant baculovirus clones were purified by one or two rounds of limiting dilution onto 96-well plates as described by Pen et al. (1989). The cells were grown for 6−8 days, and the medium from each well was saved while the cell extracts were spotted onto a nitrocellulose filter using a 96-well manifold; the nitrocellulose was then probed with ^{32}P-labeled 468 bp *Kpn*I-*Sph*I fragment of BDH-cDNA. Single clones were chosen for reamplification.

Cell Harvesting. Infected Sf 9 cells from a single T75 (75 cm^2) flask were scraped into phosphate-buffered saline [PBS, see O'Reilly et al. (1994)], pelleted at 1000*g* at 4 °C, and resuspended in 1 mL of 20 mM HEPES-NaOH, pH 8.0, 0.1 mM EDTA, with leupeptin (1 μg/mL), aprotinin (100 kallikrein inactivating units/mL), and pepstatin (1 μg/mL) added. The total protein concentration ranged from 4 to 7 mg/mL. The cells were allowed to swell for 10 min on ice in hypotonic medium and then were homogenized by passing the material through a 22 guage needle 8−10 times to lyse the cells. The material was aliquoted and stored at −20 °C temporarily or permanently at −80 °C.

Assays of BDH Activity. BDH activity was measured in a phosphate-buffered cocktail, essentially as described previously (Rudy et al., 1989; Bock & Fleischer, 1975) at 30 °C using 5 mM NAD^+ and substrate, (*R*)-3-hydroxybutyrate (HOB), added either as 20 mM R,S-HOB or as 100 mM R-HOB as indicated. BDH activity in bovine heart submitochondrial vesicles was measured in the presence of 0.5 μg of antimycin A/mL to prevent oxidation of NADH by the electron-transport system. The apparent V_{max} and K_m values for R-HOB and NAD^+ were measured essentially as described (Dalton et al., 1993) using substrate and NAD^+ concentrations as indicated and with a total Na^+ of 220 mM. For CTT-D12, the R-HOB concentration was varied up to 500 mM with 20 mM NAD^+.

Solubilization of HH-BDH from Sf 9 Cells by Digestion with Phospholipase A_2. Samples of lysed Sf 9 cells were digested with phospholipase A_2, based on the procedure for solubilizing BDH from mitochondria (Fleischer et al., 1966; McIntyre et al., 1988). Sf 9 cells were transfected with BDH-cDNA in baculovirus, harvested after 5 days' growth, and lysed in 20 mM Hepes (pH 8.0), 0.1 mM EDTA. The lysed cells were diluted to 0.64 mg of protein/mL in 25 mM imidazole-HCl (pH 7.4) and 5 mM dithiothreitol. After $CaCl_2$ was added to 3 mM, digestion was started by addition of phospholipase A_2 [*Naja naja siamensis*, partially purified from crude venom as described (Bock & Fleischer, 1975)] to 5 μg/mL. The BDH activity was measured as a function of time of digestion, and samples were removed to terminate the reaction at different times by the addition of EDTA (to 10 mM), potassium phosphate (pH 6.5, to 20 mM), and LiBr (to 0.5 M) [see McIntyre et al. (1988)]. After sedimentation of the membrane fraction at 100 000*g* for 15 min (Airfuge, Beckman Corp.), the HH-BDH released into the supernatant was assayed following preincubation with phospholipid vesicles containing PC [PC/PE/DPG (5/4/1 ratio by phosphorus), 400 μg of P/mg of protein]. For some studies, phospholipid vesicles devoid of PC (i.e., PE/DPG, 9/1 ratio

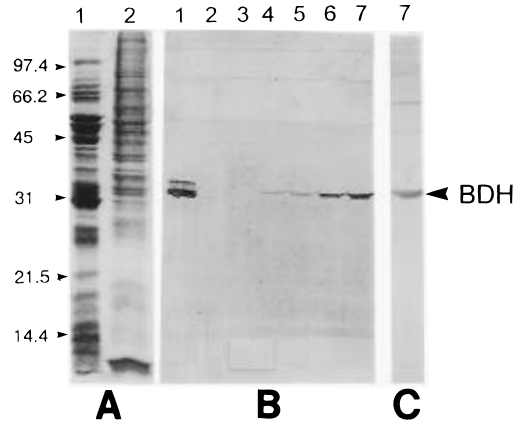

FIGURE 2: Cloned HH-BDH expressed in Sf 9 insect cells and detected by Western blot analysis. The cDNA encoding full-length HH-BDH (Marks et al., 1992) was modified to remove the leader peptide, directionally cloned into pVL1393 transfer vector, recombined into baculovirus and used to transfect Sf 9 cells derived from *S. frugiperda* (see Experimental Procedures). After 5 days' growth, cells were harvested and then lysed in 20 mM Hepes (pH 8.0), 0.1 mM EDTA with protease inhibitors (leupeptin, aprotinin, and pepstatin, 1 μg/mL), and total cell protein was separated by SDS−PAGE. Panel A. Coomassie stain. Panels B and C. Western blots detected either with anti-BDH polyclonal antibody (panel B) or anti-BDH monoclonal antibody, mAb4-4D (panel C). Samples (20 μg of protein each) are bovine heart submitochondrial vesicles (SMV) (lanes 1 in A and B); uninfected (control) Sf 9 cells (lanes 2 in A and B); Sf 9 cells transfected with a serial dilution (10×) of purified recombinant baculovirus containing BDH-cDNA (dilutions of 10^6- to 10^2-fold for lanes 3−7, respectively). The 10^2-fold dilution of virus corresponds with a multiplicity of infection of ∼5 (see Table 3). Locations of molecular mass protein standards are indicated to the left.

by phosphorus) were used instead of PC/PE/DPG vesicles so as to confirm the PC requirement for the function of HH-BDH.

Immunoblotting. Western blot analysis of lysed Sf 9 cells (infected or control) or of submitochondrial vesicles was carried out essentially as described previously (McIntyre et al., 1988; Adami et al., 1993) using SDS−PAGE with 12% polyacrylamide gels followed by transfer to polyvinylidene difluoride membrane (Immobilon-P, 0.45 μm; Millipore Corp., Bedford, MA). Immunodetection was performed with either anti-BDH rabbit polyclonal antibody or an anti-BDH mouse monoclonal antibody [mAb4-4D, which was found to be specific for a sequence epitope located in the central region of the BDH polypeptide (Adami et al., 1993)] followed by alkaline phosphatase-linked secondary antibody (anti-rabbit or anti-mouse IgG as appropriate, each from Promega Corp., Madison, WI). Color development used NBT/BCIP (Promega).

RESULTS

Infection of Sf 9 cells in culture with baculovirus containing cDNA encoding mature HH-BDH results in expression of immunodetectable polypeptide of the predicted size (31.5 kDa) which co-migrates with mature BDH present in bovine heart SMV (Figure 2). No detectable BDH protein was present in uninfected Sf 9 cells (Figure 2, lane 2). Sf 9 cells infected with increasing amounts of virus (up to a multiplicity of infection of ∼5 at 10^2-fold dilution) (Figure 2, lanes 3−7) exhibit increasing amounts of immunoreactive BDH polypeptide. A monoclonal antibody (mAb4-4D) that was prepared against BH-BDH, also detects, in infected Sf 9 cells, a

Table 1: Expression of HH-BDH Activity in Sf 9 Cells[a]

sample	protein yield (mg)	BDH specific activity (μmol min^{-1} mg^{-1}) (1) 20 mM R,S-HOB	(2) 100 mM R-HOB	BDH activity ratio, (2)/(1)
BH-SMV	−	0.58	0.24	0.41
Sf 9 cells (virus dilution)				
(10^2)	4.1	0.50	0.22	0.44
(10^3)	4.3	0.39	0.15	0.38
(10^4)	3.8	0.14	0.057	0.41
(10^5)	5.5	0.043	0.024	0.56
(10^6)	6.1	0.005	0.003	0.60
control	5.9	0	0	−

[a] Sf 9 cells were transfected at a decreasing multiplicity of infection using a 10× serial dilution of purified recombinant baculovirus containing BDH-cDNA and harvested after 5 days' growth (see Figure 2). Data are from a typical experiment using 4×10^6 cells per sample and at a multiplicity of infection of ∼5 virus/cell for 10^2 dilution of the baculovirus. The control is uninfected Sf 9 cells. BDH activity of the lysed Sf 9 cells was measured at 30 °C using 5 mM NAD^+ and either 20 mM R,S-HOB or 100 mM R-HOB as indicated. The BDH activity of bovine heart submitochondrial vesicles (BH-SMV) was measured after addition of antimycin to inhibit reoxidation of NADH by electron transport.

polypeptide of the same size as BDH (Figure 2, panel C). This mAb (prepared against BH-BDH) has been shown previously to cross-react with rat liver BDH and is specific for a segment of sequence in the central region of the polypeptide chain (between 9.0 and 16.9 kDa from the N-terminus) (Adami et al., 1993). Cross-reaction of mAb4-4D with HH-BDH is consistent with the interspecies conservation of BDH primary sequence (see Figure 1). The relative amounts of HH-BDH detected by Western blot as a function of viral dilution (Figure 2) are consistent with enzymic activity data, i.e., the transfected Sf 9 cells exhibit BDH polypeptide and enzymatic activity that decreases as the multiplicity of infection is reduced by dilution of the virus (Table 1). The enzymic activity assay to detect expressed HH-BDH in Sf 9 cells has sensitivity that is in the range of that for immunodetection of BDH by Western blot analysis (compare Figure 2 with Table 1). Measurable BDH enzymic activity can be detected in Sf 9 cells infected with a 10^6-fold dilution of virus [equivalent to a multiplicity of infection of ∼1 virus/2000 cells (Table 1)]. Thus, enzymic activity of expressed HH-BDH measured in lysates of Sf 9 cells permits detection of enzymic function that approaches 100-fold less than that for the highest level of expressed wild-type HH-BDH.

BDH activity, expressed in Sf 9 cells, requires the addition of both NAD^+ and R-HOB for assay. Comparable activity was found using either only the *R*-isomer of HOB or the racemic mixture (RS-HOB) (not shown), as found previously for BH-BDH (Dalton et al., 1993). The addition of antimycin (used in mitochondria to block electron transport and re-oxidation of NADH) did not affect the measured BDH activity in lysed Sf 9 cells, consistent with lack of a specific mitochondrial localization of HH-BDH but rather a generalized distribution throughout the membrane fraction of the cells (see below). The expressed HH-BDH activity in Sf 9 cells is inhibited by high R-HOB concentration (100 mM) with an activity ratio of ∼0.4 (Table 1), similar to inhibition of the bovine heart enzyme in SMV. For BH-BDH, the reduced activity at 100 mM R-BOH has been found to derive in large part from a generalized inhibition by high salt

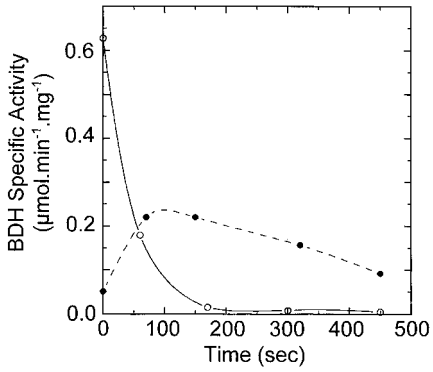

FIGURE 3: Solubilization of HH-BDH expressed in Sf 9 cells by digestion with phospholipase A_2. Sf 9 cells were transfected with BDH-cDNA in baculovirus and harvested after 5 days' growth (see Figure 2). After lysis in 20 mM Hepes (pH 8.0), 0.1 mM EDTA, cells were diluted to 0.64 mg of protein/mL in 25 mM imidazole (pH 7.4), 5 mM dithiothreitol, and 3 mM $CaCl_2$ and digestion (at 30 °C) was started by addition of phospholipase A_2 (5 μg/mL) as described previously for mitochondrial BDH (McIntyre et al., 1988). BDH activity was measured in the cells (○) and in the supernatant (●) obtained after treatment with 0.5 M LiBr followed by centrifugation and then reconstitution with PC/PE/DPG (5/4/1) vesicles (see Experimental Procedures).

concentration (Dalton et al., 1993). At the highest multiplicity of infection, the specific activity of the expressed enzyme is comparable to that of BDH in bovine heart SMV (Table 1). Several isolated subclones were tested for expression of BDH activity in whole cell lysates, and each displayed high specific activity ranging from 0.3 to 0.7 μmol min^{-1} mg^{-1} (not shown). Since BH-BDH constitutes ~1% of the protein in SMV (McIntyre et al., 1988), HH-BDH is estimated to constitute up to ~1% of the total cell protein in Sf 9 cells under these conditions (5 days' cell growth after transfection at a multiplicity of infection ~5). For a number of independently isolated and/or expressed HH-BDH clones, the Michaelis constants for NAD^+ and R-HOB for BDH catalysis showed little sample to sample variation and kinetic parameters are similar to those obtained with bovine heart BDH (Table 3). On the basis of these results, we conclude that the mature BDH polypeptide assembles in insect cells to form a catalytically active enzyme and that the "leader peptide" and mitochondrial targeting are not required for correct folding of the protein.

In Sf 9 cells, all the BDH activity and essentially all the BDH polypeptide was found in the particulate fraction (not shown) consistent with the amphipathic properties of BDH and its ability to spontaneously insert into phospholipid bilayers and natural membranes (McIntyre et al., 1988). BDH activity was measurable in whole cell lysates without the addition of exogenous PC, indicating that endogenous PC in the Sf 9 cell membranes is sufficient to fulfill the requirement for PC for activity. The requirement of lipid for the function of expressed HH-BDH was demonstrated by a striking sensitivity of activity to phospholipase A_2 digestion which results in a prompt loss of enzymic activity within several minutes (Figure 3). Following phospholipase digestion, BDH activity was restored after reconstitution with phospholipid vesicles containing PC. The maximal activity recovered in the supernatant was ~35% of the initial value [comparable to recovery of activity from bovine heart mitochondria (Bock & Fleischer, 1975; McIntyre et al., 1988)]. At longer digestion times, there was reduced recovery of activity. For both HH-BDH and BH-BDH, protein solubilized by phospholipase A_2 digestion does not exhibit enzymic activity until reconstituted with phospholipid vesicles containing PC to restore enzymic function (Table 2). Using standard assay conditions, no BDH activity was detected after reconstitution with PE/DPG vesicles devoid of PC. Therefore, the expressed HH-BDH requires phospholipid for function with a specificity for PC.

The expression of constitutively active lipid-dependent BDH in the insect cell system permitted structure−function mutagenesis studies to be undertaken. We report the first characterization of the properties of mutant forms of HH-BDH (Table 3). Two unintended PCR-derived BDH-cDNA clones (Ser24→Thr and Met92→Val) were found to produce catalytically active BDH in infected Sf9 cells. DNA sequence analysis subsequently revealed that each of these two clones contained PCR-induced nucleotide errors which

Table 2: Requirement of PC for Enzymic Activity of Solubilized HH-BDH[a]

sample	BDH specific activity (μmol min^{-1} mg^{-1}) BH-BDH in SMV	HH-BDH in Sf 9 cells
membrane bound	0.52	0.63
solubilized apoBDH	0	0
reconstituted with PL vesicles		
PC/PE/DPG	0.17	0.22
PE/DPG	0	0

[a] HH-BDH was expressed in Sf 9 cells and solubilized by digestion with phospholipase A_2 as described in Figure 3. Data for BH-BDH was obtained using BH-SMV as described previously for BH-mitochondria (McIntyre et al., 1988). After solubilization, BDH activities were measured at 30 °C (using 20 mM R,S-HOB and 5 mM NAD^+) either without addition of PL vesicles or after 15 min incubation (30 °C) with either PC/PE/DPG (5/4/1) or PE/DPG (9/1) vesicles (400 μg of P/mg of total cell protein in the digest).

Table 3: Enzymic Function of Cloned BDH and Mutants[a]

BDH sample	V_{max} (μmol min^{-1} mg^{-1})	$K_m^{NAD^+}$ (mM)	K_m^{HOB} (mM)
bovine heart (in SMV)	0.80 ± 0.15	0.95 ± 0.15	0.50 ± 0.11
human heart (in Sf 9)			
unmodified	0.83 ± 0.05	0.53 ± 0.08	0.71 ± 0.11
Met92→Val mutant	(0.6 ± 0.1)	ND[b]	ND
Ser24→Thr mutant	0.5 ± 0.1	0.6 ± 0.2	0.7 ± 0.2
Cys242→Ser mutant	0.8 ± 0.2	0.8 ± 0.2	10 ± 2
CTT-D12[c]	<0.1	ND	>100

[a] Enzymic function of either BH-BDH (in SMV) or the mature form of HH-BDH (unmodified or mutants each expressed in Sf 9 cells, see Figure 4) were measured at 30 °C (see Experimental Procedures). The specific activities were measured as a function of R-HOB and NAD^+ to determine the V_{max} and the Michaelis constants for NAD^+ and R-HOB (BDH-catalyzed oxidation of R-HOB) using for K_m^{HOB}, 20 mM NAD^+ with 0.5−10 mM R-HOB except for the Cys242→Ser variant where 2−100 mM R-HOB was used; for $K_m^{NAD^+}$, 0.1−20 mM NAD^+ with 10 mM R-HOB except the Cys242→Ser variant for which 100 mM R-HOB was used. Values given are the mean ± SEM from at least three independent determinations. For the Met92→Val mutant, kinetic parameters were not determined; the value given in the V_{max} column is the specific activity at 20 mM R,S-HOB with 5 mM NAD^+. [b] ND, not determined. [c] CTT-D12 refers to the variant of HH-BDH with the twelve C-terminal amino acids deleted (see Figure 1). For CTT-D12, no activity was detected at the limit of sensitivity of the assay (equivalent to a specific activity of <0.002) using 20 mM R,S-HOB. At higher substrate concentrations, the CTT-D12 variant exhibited low enzymic activity (0.01−0.02 μmol min^{-1} mg^{-1}; 100 mM R-HOB, 20 mM NAD^+) and increased activity (up to 0.05 μmol min^{-1} mg^{-1}) at 500 mM R-HOB, indicating that K_m^{HOB} is >100 mM for CTT-D12.

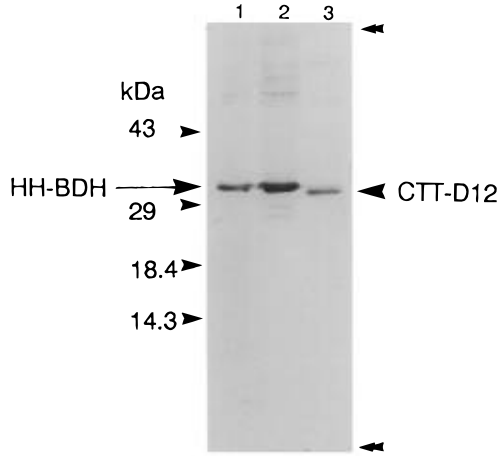

FIGURE 4: Expression of HH-BDH and two variants in Sf 9 cells as detected by Western blot. Sf 9 cells were infected with baculovirus containing BDH-cDNA encoding either the unmodified mature form of HH-BDH (lane 1, "wild-type") or the Cys-242→ser variant (lane 2) or CTT-D12 variant (12 amino acids deleted from the C-terminal) (lane 3). After 5 days' growth, cells were harvested, lysed, and analyzed by Western blot using anti-BDH polyclonal antibody (see Figure 2). Double arrowheads denote the top and bottom of the gel.

resulted in single amino acid substitutions, one in each of the two isolates as follows: Ser24 changed to Thr, and Met92 changed to Val. Both of these variants exhibited BDH activity comparable to the unmodified enzyme (Table 3), indicating that these residues are not vital for function. These conclusions were validated by correction of the unintended nucleotide errors and characterization of the expressed wild-type HH-BDH (see Table 3).

Peptide sequence (Yamaguchi et al., 1986) indicates that the reactive sulfhydryl of bovine heart BDH corresponds with Cys242 of HH-BDH, the only cysteine in the C-terminal (nonhomologous) domain of BDH (Marks et al., 1992). The corresponding residue in bovine heart BDH reacts with arylazido-NAD (Yamaguchi et al., 1986). Since the predicted NAD(H)-binding fold of BDH resides in the N-terminal domain, the N-terminal and C-terminal domains of BDH appear to be in proximity. The role of Cys242 in the function of HH-BDH was explored by using site-directed mutagenesis to change Cys to Ser. The Cys242→Ser variant was expressed in Sf 9 cells to levels similar to the unmodified HH-BDH (Figure 4). The substitution of serine for Cys242 results in a ~10-fold increase in the apparent K_m^{HOB} with no significant change in the apparent K_m^{NAD} (Table 3). Thus, Cys242 is important for substrate binding to BDH but has no effect on the binding of NAD^+, as reflected in the K_m^{NAD} being unaltered.

We have shown that carboxypeptidase cleavage of only a few ($\leq$14) C-terminal amino acids from BH-BDH prevents activation by PC (Adami et al., 1993). Further, for BDH reconstituted in bilayer lipids containing PC, the C-terminus is protected from enzymatic digestion with carboxypeptidase, an effect not observed in the absence of PC in the bilayer (Berrez et al., 1984; McIntyre et al., 1990; Adami et al., 1993). These data suggest that in the presence of activating PC, the C-terminus of BDH interacts with the lipid. These results are in accord with analysis of the amino acid sequence of HH-BDH which predicts that lipid binding and activation by PC are conferred by the C-terminal domain of the protein (Marks et al., 1992). A deletion clone of HH-BDH was constructed to remove twelve amino acids from the C-terminal of the mature HH-BDH. This construct (CTT-D12) is expressed in Sf 9 cells (Figure 4). As expected, immunodetectable BDH, expressed from CTT-D12, migrates with a slightly faster mobility on SDS−PAGE. The difference in size of the truncated CTT-D12 detected in the Western blot is consistent with deletion of twelve amino acids. The expressed CTT-D12 exhibits no detectable enzymic activity using 20 mM R,S-HOB (Table 3, footnote *b*). On the basis of the sensitivity of the enzymic assay, CTT-D12 samples have a BDH specific activity that is <0.002 μmol NAD^+ reduced min^{-1} mg^{-1} at 20 mM R,S-HOB; this is at least 100-fold lower than any other clone containing expressed BDH. At high substrate concentration, Sf 9 cells expressing the truncated CTT-D12 variant exhibited detectable BDH activity (see footnote *b*, Table 3), suggesting that this truncated HH-BDH variant may be folded correctly despite lack of the twelve residues at the C-terminal.

DISCUSSION

The full-length cDNA for the mature form of HH-BDH has been expressed in insect cells in a constitutively active form. The results show that the leader sequence for mitochondrial targeting of BDH is not required for expression of active enzyme. Further, HH-BDH is shown to have a lipid requirement analogous to bovine heart and rat liver BDH, i.e., PC is required for the function of HH-BDH. For the expressed enzyme, the endogenous lipid in the insect cells fulfills the lipid requirement. That HH-BDH has kinetic parameters similar to those for bovine heart BDH (Table 3) is consistent with previous studies showing similar enzyme kinetic parameters for the rat liver and bovine heart enzymes (McIntyre et al., 1988). A previous study from Churchill's group achieved expression of a truncated form of rat liver BDH (seven N-terminal amino acids absent) that was active only after reconstitution with phospholipid vesicles (Jones et al., 1993). The studies reported here are the first demonstration of expression from BDH-cDNA of a full-length mature form of BDH in constitutively active form.

Expression of constitutively active BDH permitted structure−function mutagenesis studies of BDH to be carried out directly in the insect cells. For such studies, enzymic function can be detected at levels approaching 100-fold less than that for optimally expressed wild-type HH-BDH (see Table 1). The first site-directed mutants of HH-BDH have been prepared and expressed in insect cells (Figure 4 and Table 3). Three of the mutants are active. For the Met-92→Val variant, this non-conservative substitution has no effect on activity and therefore this Met is clearly nonessential although it is in the vicinity of a highly conserved segment (LVNNAGI, residues 95−101) (Persson et al., 1991) of the substrate/catalytic site domain (see Figure 1). For the Ser24→Thr mutant [a mutation in the NAD(H) binding domain, see Figure 1], the measured enzymic function is similar to the unmodified HH-BDH (Table 3). Since the enzyme has an ordered sequential reaction mechanism in which NAD^+ binds first (Nielsen et al., 1973), the K_m^{NAD} reflects the binding of NAD^+ to the enzyme (provided the substrate is saturating). The K_m^{NAD} for the Ser24→Thr mutant and unmodified HH-BDH are the same within error. Thus, the conservative substitution of Ser24 by Thr, i.e., the addition of a methyl group at this locus, does not affect function, although this residue is between two residues (e.g.,

Gly22, Ala26) that are highly conserved in the NAD(H) binding domain of the family of SC-ADH.

The mature form of HH-BDH has five cysteine residues which are conserved in the rat liver enzyme (see Figure 1). Bovine heart and rat liver BDH each have a total of six cysteines (Latruffe et al., 1980; Churchill et al., 1992), which in the bovine heart enzyme exist as two sulfyhydryls (SH1 and SH2) and two disulfide bridges (Fleer et al., 1984). Chemical derivatization studies have previously shown that the more reactive sulfhydryl (SH1) of the bovine heart enzyme is essential for optimal function (Latruffe et al., 1980), but it does not appear to be involved in catalysis (Dubois et al., 1986). Selective derivatization of SH1 of bovine heart BDH with reagents of different sizes resulted in reduced activity that correlated with the size of the reagent. With SH1 derivatized, there was a marked increase in the K_m^{HOB} (up to 100-fold for the diamide derivative of BDH) with a smaller increase (2−5-fold) in the K_m^{NAD} (Dubois et al., 1986; Dalton et al., 1993) suggesting that Cys242 may be in the vicinity of the substrate and/or catalytic site. Results obtained with the Cys242→Ser mutant show that the sulfhydryl is not required for NAD^+ binding since the K_m^{NAD} is the same within error as for wild-type HH-BDH (Table 3). By contrast, this mutant displays an ∼10-fold increase in the K_m^{BOH}, which is comparable to that afforded by cyanylation of SH1 of bovine heart BDH (Dubois et al., 1986). That the replacement of a sulfur by oxygen in the Cys242→ser variant has such a profound effect on the K_m^{BOH} suggests that this sulfhydryl is involved either directly or indirectly in substrate binding.

Deletion of the twelve C-terminal amino acids from HH-BDH in the CCT-D12 construct results in loss of enzymic function as measured using physiologically relevant substrate concentrations (Table 3). Carboxypeptidase cleavage of ≤14 C-terminal amino acids previously indicated a role for the C-terminus in lipid binding (Adami et al., 1993). The twelve C-terminal residues are clearly essential for function since the truncated CTT-D12 mutant of BDH is inactive at physiologically relevant concentrations of HOB and NAD^+. For this truncated mutant BDH, high substrate concentration afforded limited catalysis. We previously found that, although purified BH-BDH, in the absence of PC, has no activity using the standard substrate concentration (10 mM R-HOB in the racemic R,S-HOB mixture) (Fleischer et al., 1966; Grazzotti et al., 1974; Bock & Fleischer, 1975; Grover et al., 1975), some catalytic activity can be detected at high substrate concentration (100 mM R-HOB) (Rudy et al., 1989; McIntyre et al., 1990). That CTT-D12 exhibits detectable catalytic activity at high substrate indicates a reduced substrate affinity for this truncated form of BDH, which appears to be analogous to the low substrate affinity for BDH devoid of PC (Rudy et al., 1989; McIntyre et al., 1990). Results obtained with the CTT-D12 variant confirm that the C-terminus is important for the function of BDH and are consistent with a role for this domain of the protein in lipid binding as is required for optimal BDH catalysis.

A number of enzyme systems are known to be lipid dependent [see Fleischer and McIntyre (1985) and Sandermann et al. (1993)]. For BDH, the mechanism of lipid activation has remained elusive despite detailed characterization of the nature of the lipid requirement and its effect on activity [albeit see Gazzotti et al. (1974)]. We postulate, on the basis of sequence analysis (Marks et al., 1992) and proteolysis studies (Berrez et al., 1984; Maurer et al., 1975; Adami et al., 1993), that the C-terminal domain of BDH is involved in lipid binding and probably the activation by PC. By contrast, the activation of protein kinase C by phosphatidylserine appears to require the N-terminal domain for binding to the lipid. It is postulated that the binding of lipid induces an allosteric change in protein kinase C involving a pseudosubstrate binding domain so as to allow the interaction of substrate with the active site (Newton, 1993). By analogy, a similar autoinhibitory domain may be present in the C-terminus of BDH although a variety of other mechanisms can be conceptualized to account for the PC-induced allosteric activation of the enzyme. Since the truncated CTT-D12 variant has minimal activity, it would appear that the twelve C-terminal amino acids do not constitute such an autoinhibitory domain in HH-BDH.

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