Phosphatidylcholine Activation of Human Heart (*R*)-3-Hydroxybutyrate Dehydrogenase Mutants Lacking Active Center Sulfhydryls: Site-Directed Mutagenesis of a New Recombinant Fusion Protein†

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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. A plasmid has been constructed to express human heart (HH) BDH in *Escherichia coli* as a hexahistidine-tagged fusion protein (HH-Histag-BDH). A rapid two-step affinity purification yields active HH-Histag-BDH (and six mutants) with high specific activity (∼130 μmol of NAD^+ reduced·min^{-1}·mg^{-1}). HH-Histag-BDH has no activity in the absence of phospholipid and exhibits a specific requirement of PC for function. The HH-Histag-BDH−PC complex (and HH-BDH derived therefrom by enterokinase cleavage) has apparent Michaelis constants (K_m values) for NAD^+, NADH, (*R*)-3-hydroxybutyrate (HOB), and acetoacetate (AcAc) similar to those for bovine heart or rat liver BDH. A computed structural model of HH-BDH predicts the two active center sulfhydryls to be C69 (near the adenosine moiety of NAD) and C242. With both sulfhydryls derivatized, BDH has minimal activity, but site-directed mutagenesis of C69 and/or C242 now shows that neither of these cysteines is required for PC activation or catalysis (the double mutant, C69A/C242A, is highly active with essentially normal kinetic parameters). Six cysteine mutants each have an increased K_m^{NADH} (2−6-fold) but an unchanged $K_m^{NAD^+}$. The C242S and C69A/C242S enzymes (but not the analogous C242A mutants nor the C69A or C69S mutants) exhibit ∼10-fold increases in K_m^{HOB} and K_m^{AcAc}, reflecting an altered substrate binding site. Thus, although C242 (in the C-terminal lipid binding domain of BDH) is close to the active site, it appears to be in a hydrophobic environment and only indirectly defines the substrate binding site at the catalytic center of BDH.

(*R*)-3-Hydroxybutyrate dehydrogenase (BDH;[1] EC 1.1.1.30) is a mitochondrial lipid-requiring enzyme with a specific requirement of phosphatidylcholine (PC) for function (*1*−*3*). BDH has been purified and characterized from bovine heart (BH-BDH) (*4*) and is a paradigm for lipid−protein interactions in membranes. BDH purified from the rat, either liver (*3*) or brain (*5*), has properties comparable to those of BH-BDH including a similar requirement of PC for catalysis. The cDNA for BDH, cloned from both human heart (HH) (*6*) and rat liver (*7*), encodes the precursor form of the protein including an N-terminal leader peptide that serves to target BDH to the matrix face of the mitochondrial inner membrane (*8*). Cleavage of the leader peptide yields the 297-residue mature form of the enzyme, mBDH, that for human and rat is ∼90% identical in amino acid sequence (as specified by their cDNAs) (see ref *9*). Sequence homology identifies BDH (*6*, *7*) as a member of the short-chain (nonmetalloenzyme)

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[1] Abbreviations: AcAc, acetoacetate; BDH, (*R*)-3-hydroxybutyrate dehydrogenase; BH, bovine heart; buffer A, 16 mM sodium phosphate buffer (pH 7.4) with 10 mM β-mercaptoethanol; buffer B, 25 mM HEPES−NaOH (pH 8.3) containing 10 mM DTT and 25 mM NaCl; DPG, diphosphatidylglycerol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HH, human heart; HOB, hydroxybutyrate; Histag, 17-residue N-terminal fusion peptide containing a hexahistidine sequence and enterokinase cleavage site; HH-Histag-BDH, fusion protein of HH-BDH with Histag; IPTG, isopropyl β-D-thiogalactoside; K_m^{HOB}, K_m^{AcAc}, $K_m^{NAD^+}$, and K_m^{NADH}, apparent Michaelis constants for HOB, AcAc, NAD^+, and NADH, respectively; PC, phosphatidylcholine; PCR, polymerase chain reaction; PE, phosphatidylethanolamine; SC-ADH, short-chain alcohol dehydrogenase (*10*).

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alcohol dehydrogenase (SC-ADH) family of proteins (*10*−*12*). This homology, together with the 3D structure of two related proteins of this family (*13*, *14*), predicts the N-terminal two-thirds of BDH to constitute the catalytic domain of the enzyme, with the first ∼90 residues forming the coenzyme binding site (*6*, *9*). The C-terminal third of the BDH sequence (residues 194−297) is unique and was predicted to confer phospholipid binding and the specific activation by PC (*6*). An important advance in understanding the role for PC in activating BDH was the finding of a PC requirement for NADH binding to the enzyme (*15*). This subsequently was shown to be referable to a PC-induced enhancement in NAD(H) binding to BDH, i.e., an ∼50-fold PC-induced reduction in the K_D for NAD(H) (*16*). Likewise, the binding of NAD(H) to BDH enhances the interaction of PC with the protein, reflecting allosteric interaction between its PC and NAD(H) binding sites (*17*).

BDH has six cysteines that, in BH-BDH, exist as two sulfhydryls (referred to as SH1 and SH2) and two disulfides (*18*). Although the catalytic site of the SC-ADH contains a tyrosine and a lysine but not a cysteine (*19*), sulfhydryls do serve important roles in a number of redox reactions, e.g., in catalysis by the aldehyde dehydrogenases (*20*). The strict conservation of the cysteines in the three species (human, bovine, and rat) of BDH, for which amino acid sequences have been reported (*6*, *9*), suggests the possibility that these residues may play a role in the structure, function, or PC activation of this enzyme. Indeed, chemical modification studies had indicated a role for cysteine in BDH catalysis (*21*, *22*) and, subsequently, that both sulfhydryls of BH-BDH may be in the vicinity of the active center (*23*, *24*). The concomitant derivatization of both sulfhydryls yields an enzyme with minimal catalytic function (*23*, *24*), implying that BDH may require at least one sulfhydryl in the active center for PC activation and/or optimal catalysis. Recombinant forms of rat liver BDH have been expressed (*25*, *26*), and we reported the expression, in Sf9 cells, of constitutively active full-length recombinant human heart BDH (*9*). A C242S mutant of BDH that was expressed in Sf9 cells exhibited an ∼10-fold increase in its K_m^{HOB}, suggesting a role for this cysteine in substrate binding. In this report, a plasmid has been designed and constructed so as to express, in *Escherichia coli*, a hexahistidine-tagged fusion protein (HH-Histag-BDH) containing a fully functional form of HH-BDH that provides a platform for structure−function mutagenesis studies. HH-Histag-BDH and a set of six single or double site-directed cysteine mutants of this recombinant fusion protein now have been expressed, purified, and characterized. The results show, for the first time, that BDH mutants lacking either or both of the active center sulfhydryls, C69 or C242, each require PC for function and have catalytic activities comparable to those for unmodified BDH that contains both of these cysteines. In the absence of DTT, the mutants that lack C242 are found to have increased stability against aerobic inactivation. It is further shown that two purified C242S mutants of HH-BDH exhibit increased apparent Michaelis constants for both the reduced and oxidized substrates [confirming and extending the prior study of the C242S mutant expressed in Sf9 cells (*9*)]. By contrast, the analogous C242A mutants and the C69A or C69S mutants are found to have enzyme kinetic parameters and activities similar to those of unmodified BDH, refuting a possible direct role for either of its sulfhydryls in either substrate binding or catalysis.

EXPERIMENTAL PROCEDURES

Oligonucleotide Primers. The oligonucleotides used in the construction of the HH-Histag-BDH expression plasmid had the following sequences: GG***GGTACC***AGGGTATTAATAATGAAAGGGAATCATCACCATCACCACC (primer 1) and CTCC***GCCGCACTGGC***TTTGTCATCGTCGTCGCCACCATGGTGGTGATGGTGATG (primer 2). Complementary nucleotides are underlined and the *Kpn*I and *Bgl*I sites (in primers 1 and 2, respectively) are in bold italics. Mutagenic oligonucleotides (used for site-directed mutagenesis of Cys69 and Cys242) had the following sequences: TTCGCTGCT**AGC**GACATTGAG (primer 3, C69A, antisense), TTCGCTGCT**GGA**GACATTGAG (primer 4, C69S, antisense), and GGAGACCTAC**T/GCT**AGCAGTGGCT (primer 5, C242A,S). Positions of mutations are shown in bold.

Construction of HH-Histag-BDH Expression Plasmid pBDH-VU1. Cloning experiments were performed using *E. coli* K12 JM109 (*27*) and standard methods (*28*). In brief, to construct pBDH-VU1, primer 1 and primer 2 were annealed, and double-stranded (blunt-end) DNA was prepared with T4 DNA polymerase and dNTP. The resulting double-stranded fragment (total of 86 base pairs) was used to create a new sequence encoding 17 additional N-terminal amino acids upstream to mBDH−cDNA. From two separate endonuclease digests (*Pae*I−*Bgl*I and *Kpn*I−*Pae*I, respectively) of mBDH-Bluescript, the pBlueScript vector containing the cDNA for mature human heart BDH (*9*), two DNA fragments (one of 445 bp and the other of 3687 bp) were purified (see Figure 1). The 86-residue double-stranded DNA fragment was digested with *Kpn*I and *Bgl*I, and this was then ligated together with the two DNA fragments purified from mBDH-Bluescript. The resulting plasmid (pBDH-KL1) was then cleaved with *Kpn*I and *Eco*RI to obtain the DNA encoding the new fusion protein, HH-Histag-BDH (with hexahistidine and an enterokinase cleavage site N-terminally fused to the mature HH-BDH sequence); this DNA was ligated into the multiple cloning site of pUC119 (Clontech, Palo Alto, CA) (*29*), yielding the expression plasmid pBDH-KL2. A spurious mutation in the BDH−cDNA sequence [identified previously as a PCR-induced M92V mutation (*9*)] was corrected as before [i.e., the *Eco*NI fragment from the original full-length BDH−cDNA in BlueScript SK(−) (*6*) was exchanged for the *Eco*NI fragment in pBDH-KL2], yielding the HH-Histag-BDH expression plasmid pBDH-VU1 (see Figure 1).

The insert encoding the HH-Histag-BDH in pBDH-VU1 was sequenced and revealed three discrepancies in the published cDNA sequence of HH-BDH (*6*, *9*) as follows: residue 344 is G, residue 348 is C, and residue 746 is G (residue numbers as in ref *6*); the same sequence was found in the original clone, mBDH-Bluescript (*9*). The G found at residue 344 is consistent with the observed two-site cleavage of HH-BDH-cDNA by *Pst*I (not shown). These corrections in cDNA sequence for HH-BDH result in three changes in the predicted amino acid sequence of HH-BDH: at residues 69 (Cys not Phe), 70 (Ser not Arg), and 203 (Ser not Asp). The corrected residues, C69 and S203, are identical to the corresponding residues in rat liver BDH (*7*).

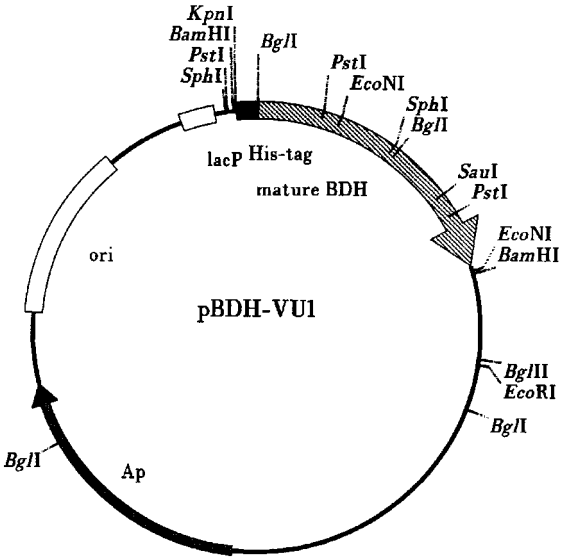

FIGURE 1: Human heart (*R*)-3-hydroxybutyrate dehydrogenase expression plasmid pBDH-VU1. From pUC119, a two-step procedure (see Experimental Procedures) was used to create the expression vector, pBDH-VU1, that includes the cDNA coding for mature HH-BDH (mBDH) (crosshatched arrow) with a new 5′ end (as indicated in bold, *Kpn*I to *Bgl*I sites) designed to code the cleavable Histag. pBDH-VU1 codes expression of HH-Histag-BDH (see Table 1). Ap identifies the ampicillin resistance gene (bla) specifying β-lactamase; ori is the replicative origin; and lacP is the lac promoter, regulated by the lac repressor and inducible with IPTG. Positions of a number of restriction enzyme cleavage sites are illustrated.

Site-Directed Mutagenesis. The substitution of C69 or C242 for either serine or alanine was achieved by oligonucleotide-directed mutagenesis (*30*) of HH-Histag-BDH using the negative selection of uracil-containing template DNA (*31*). The *E. coli* strains used for mutagenesis were TG1 (*32*) and CJ236 (*31*). Appropriate subclones of pBDH-VU1were mutagenized in M13mp18 (*27*), the inserted DNA was sequenced, and the respective mutant inserts were then recloned into the pBDH-VU1 vector. Each mutation was confirmed by digesting the plasmids with *Pst*I (one *Pst*I cleavage site was removed by each mutation) and by DNA sequence. No cloning-induced sequence errors were found in any of the four expression plasmids (identified as pBDH-A69, pBDH-S69, pBDH-A242, and pBDH-S242) for the four single mutants, C69A, C69S, C242A, and C242S, respectively. Plasmids expressing the double mutations, C69A/C242A and C69A/C242S, were constructed by combining (cassetting) appropriate segments of the corresponding single mutant plasmids. Other than the single or double mutations, these plasmids are otherwise identical with pBDH-VU1.

Expression and Purification of HH-Histag-BDH. *E. coli* JM109 was used for expression of HH-Histag-BDH from pBDH-VU1. Cultures (4 × 500 mL) were grown at 37 °C on a shaking incubator in LB medium (1% Bactotryptone, 0.5% yeast extract, 1% NaCl) supplemented with 50 μg of ampicillin/mL. At an optical density of 0.6 (at 560 nm), isopropyl β-D-thiogalactoside (IPTG) was added to 1 mM, and incubation was continued for 6 h. Cells were harvested by centrifugation (8670*g*) for 10 min, washed, and resuspended in 40 mL of buffer A [16 mM sodium phosphate buffer (pH 7.4) with 10 mM β-mercaptoethanol] plus 40 mM imidazole. The cells were broken using two passes through a French pressure cell (Aminco Instruments, Champaign, IL) at 8000−10 000 psi (~300 kPa) to obtain a lysate, which was then treated with RNase I and DNase I (final concentration of 1 μg/mL for each enzyme) for 10 min at 4 °C. Following addition of phenylmethanesulfonyl fluoride (PMSF) (1 mM, final concentration), the lysate was centrifuged at 48400*g* for 20 min. PC-dependent BDH activity was found in the supernatant (see assay below). This lysate supernatant was loaded onto a 1 mL His-Trap column (Pharmacia Biotech, Piscataway, NJ). The column was washed with buffer A, containing 100 mM imidazole, and the enzyme was eluted with 500 mM imidazole in buffer A, collecting 1 mL fractions. Fractions containing BDH activity were pooled and dialyzed for 1 h against 500 mL of buffer B [25 mM HEPES−NaOH (pH 8.3) containing 10 mM DTT and 25 mM NaCl]. This dialyzed sample was applied to a 6 mL DEAE-Sepharose CL-6B (Pharmacia-Biotech, Piscataway, NJ) column that was preequilibrated and eluted with buffer B; BDH activity eluted in the void volume. Fractions containing BDH activity were pooled and brought to pH 7.5 with 0.5 M HEPES buffer (pH 6.8), and 4 M NaCl solution was then added to a final concentration of 0.5 M. The purified HH-Histag-BDH sample was concentrated using an Amicon cell with PM-10 membrane (Amicon Corp., Bedford, MA) (see ref *3*) to about 1 mg of protein/mL, frozen, and stored in a liquid nitrogen refrigerator. The purification procedure, from lysis of cells to freezing of the purified sample, takes about 7 h. Similar procedures were used to express and purify the set of six cysteine mutants of HH-Histag-BDH (C69A, C69S, C242A, C242S, C69A/C242A, and C69A/C242S).

Enterokinase Cleavage of HH-Histag-BDH. The 17-residue N-terminal fusion peptide (Histag), containing the enterokinase recognition sequence DDDDK, was cleaved from HH-Histag-BDH by digestion with enterokinase (obtained as a 1 unit/μL solution from Stratagene, Inc., San Diego, CA), yielding HH-BDH. HH-Histag-BDH (at 0.2 mg/mL) was incubated for 1 h at room temperature (22 °C) with enterokinase (20 units/mg of BDH) in 20 mM HEPES−NaOH, pH 8.0, 1 mM EDTA, and 10 mM DTT. Under the same conditions, controls of BH-BDH [purified as described previously (*3*)] were also treated with enterokinase. The undigested and digested proteins (0.75 μg each) were analyzed by SDS−PAGE with Coomassie Blue staining. Alternatively, for preparative digestion, HH-Histag-BDH (2 mg/mL in 0.5 M NaCl, 20 mM HEPES−NaOH, pH 6.7, and 10 mM DTT) was treated with enterokinase (10 units/mg of BDH) at room temperature under argon, resulting in complete cleavage within 2 h (not shown). For enterokinase treatment of HH-BDH expressed in Sf9 cells (*9*), similar conditions were used except that total protein was 2 mg/mL and HH-BDH was detected by Western blot (data not shown).

Phospholipid Activation of (R)-3-Hydroxybutyrate Apodehydrogenase. In general, the activity of HH-Histag-BDH was measured after preincubation with phospholipid vesicles using the complex assay method (*3*). Phospholipid vesicles [either PC alone or codispersions of PC/PE/DPG (5:4:1 ratio by phosphorus)] were prepared as described (*3*) using dioleoyl-PC, dioleoyl-PE, and bovine heart DPG (from Avanti Polar Lipids, Alabaster, AL). In brief, a complex of the enzyme with phospholipids was reconstituted by mixing the apoenzyme with different types and/or amounts of

aqueous microdispersions of phospholipid vesicles (as indicated). The mixture was incubated at either 23 or 37 °C in reconstitution buffer (20 mM Tris-HCl, pH 8.1, 1 mM EDTA, 5 mM dithiothreitol, and 5 mM NAD^+) with HH-Histag-BDH generally being 0.1−0.2 mg/mL in a total volume of 60 μL; after 15 min preincubation, BDH activity of an aliquot was determined (see below). Incubation of the enzyme−phospholipid mixture for up to 2 h did not increase enzymatic activity at any of the phospholipid/protein ratios tested. For BDH assays of fractions obtained during purification, activity was measured using the cuvette assay in which the enzyme was reactivated by preincubation with PC/PE/DPG (5:4:1) vesicles in the cuvette for 15 min prior to initiating the reaction with substrate (*3*).

Stability of HH-Histag-BDH and Mutants. To test the stability of HH-Histag-BDH or selected C69 and C242 mutants, the enzymes were reconstituted with PC vesicles (200 μg of lipid phosphorus/mg of protein, ∼200 mol/mol of BDH), dialyzed under argon versus 20 mM HEPES−NaOH (pH 7.0 at 4 °C) and 1 mM EDTA, and then incubated at ∼0.1 mg of protein/mL on ice in air (i.e., without argon) in either the presence or absence of 10 mM DTT. At intervals over several days, aliquots of each sample were removed and assayed for BDH activity in the standard mix (see below) except without DTT in the assay medium. For each enzyme sample, the specific activities were the same before and after dialysis and in the presence versus absence of DTT in the assay.

Analytical Methods. Protein was measured by the method of Lowry (*33*) as described by Ross and Schatz (*34*), with bovine plasma albumin as standard. Phosphorus was measured using a modification (*35*) of the method of Chen et al. (*36*). BDH activity was measured at either 30 or 37 °C (as indicated) in a phosphate-buffered cocktail (*4*) as modified (*16*) using, in general, 5 mM NAD^+ and 20 mM (*R,S*)-3-hydroxybutyrate (HOB) with 0.3 mM DTT. For measuring apparent K_m values, the *R*-isomer of the substrate [(*R*)-HOB] was used. Activities measured at 30 °C are approximately half the activity at 37 °C [an activity ratio similar to that observed with bovine heart and rat liver BDH (*3*)]. For measuring the apparent K_m for (*R*)-HOB (K_m^{HOB}), the NAD^+ concentration was 10 mM with (*R*)-HOB varied either from 0.3 to 10 mM or from 0.3 to 50 mM (as indicated). For measuring the apparent K_m for NAD^+ ($K_m^{NAD^+}$), the (*R*)-HOB concentration was 10 mM with NAD^+ varied from 0.3 to 10 mM. For measuring the apparent K_m for AcAc (K_m^{AcAc}), the NADH concentration was 0.2 mM with AcAc varied from 0.25 to 5 mM. For measuring the apparent K_m for NADH (K_m^{NADH}), the AcAc concentration was 4 mM with NADH varied from 0.005 to 0.20 mM. For these studies, the reaction was started by addition of the preformed enzyme−phospholipid complex [prepared with PC/PE/DPG (5:4:1) vesicles at 100 mol of PC/mol of BDH; see above] to the assay mixture. SDS−PAGE on 12% polyacrylamide gels (*37*) was as described (*38*).

Molecular Modeling. The programs QUANTA (version 4.0) and CHARMm (version 23.2) were from Molecular Simulations (San Diego, CA). The structure of HH-BDH was modeled with QUANTA 4.0 according to the HH-BDH sequence [(*6*, *9*) as corrected in this work] and the coordinates of the homologous 3α,20β-hydroxysteroid dehydrogenase (PDB code 2hsd) (*19*). Missing residues (insertions in HH-BDH) and different side chain atoms were built by the program as necessary. At sites of deletions or insertions, the backbone was relaxed by regularization (minimization). The complete HH-BDH structure, as derived from QUANTA, was then minimized in the CHARMm force field by the adopted base Newton−Raphson algorithm to an energy gradient tolerance of 0.01 kcal/(mol·Å). In the lipid binding domain of HH-BDH (residues 194−297), there is little homology with the 3α,20β-hydroxysteroid dehydrogenase, and therefore, the predicted structure in this region may represent a local minimum; with no secondary structure being identified in this domain, its structure is considered to be poorly defined. NAD was inserted into the energy-minimized model of BDH on the basis of its location in the SC-ADH (*19*) since the NAD site is very well defined by the conserved domains of the protein.

RESULTS

The expression plasmid, pBDH-VU1 (Figure 1), has been constructed to express a novel fusion protein (HH-Histag-BDH) consisting of the mature form of human heart BDH fused at its N-terminus with a 17-residue peptide that includes a hexahistidine tag and an enterokinase cleavage site. Upstream from the HH-Histag-BDH start codon, the pBDH-VU1 plasmid includes a translation initiation sequence modeled after the *E. coli lpp* sequence (*39*). DNA sequencing revealed three differences in pBDH-VU1 compared with the published sequence of HH-BDH (*6*, *9*). The corrected DNA sequence (see Experimental Procedures) yields a corresponding amino acid sequence of mature BDH with changes at residues 69, 70, and 203 (Cys, Ser, and Ser, respectively). The corrected amino acid sequence increases (to 90%) and reconfirms the close homology of the rat liver and the human heart forms of the enzyme and establishes a three-species (human, bovine, and rat) conservation for all six cysteines of BDH (*7*, *18*).

HH-Histag-BDH could be expressed, after IPTG induction, to about 0.4% of the total protein in the lysate (as estimated from the specific enzymic activity measured after reconstitution with phospholipid vesicles containing PC; see Table 1). Up to about 80-fold purification was achieved in a single step by nickel affinity chromatography over a His-Trap column. Further purification was achieved by subsequent DEAE-Sepharose chromatography. A typical purification is summarized in Table 1 and takes about 7 h from lysis of the cell pellets to freezing of the purified sample. Fractions from each stage of purification were analyzed by SDS−PAGE (Figure 2). For HH-Histag-BDH, the size by SDS−PAGE is in accordance with the size calculated from the sequence (35 kDa). The specific BDH activity of the purified HH-Histag-BDH (Table 1) is comparable to that for the purified bovine heart and rat liver enzymes (see ref *3*).

HH-Histag-BDH, expressed from the plasmid pBDH-VU1, has an N-terminal amino acid sequence of MKGNHHHH-HHGGDDDDK**ASAA** (the first four amino acids of HH-BDH are shown in bold); the 17-residue leader includes N-terminal methionine linked to the hexahistidine tag followed by the DDDDK enterokinase binding motif (*40*). The enterokinase cleavage site is on the C-terminal side of lysine (the second K of HH-Histag-BDH adjacent to the first Ala of HH-BDH), yielding a protein with the N-terminus, ASAA..., as in the mature form of HH-BDH (*6*). The

Table 1: Purification of HH-Histag-BDH Expressed in *E. coli*[a]

fraction[a]	vol (mL)	BDH act.[b] (μmol min^{-1})	protein (mg)	BDH sp act. (μmol min^{-1} mg^{-1})	recovery act. (%)	purification (*x*-fold)
lysate supernatant	40	193	800	0.25	100	
affinity purified	5	140	7.3	19	73	80
DEAE	1.0	78	1.2	65[c]	40	260

[a] Results shown are for a typical purification (see Experimental Procedures) from a 2 L culture of *E. coli* infected with the pBDH-VU1 expression plasmid for HH-Histag-BDH (Figure 1). Lysate supernatant refers to the 48400*g* supernatant from the lysate obtained by breaking the *E. coli* cells with a French pressure cell. The affinity-purified fraction is the pooled active fractions after affinity purification over the His-Trap column. The DEAE fraction is obtained after DEAE-Sepharose CL-6B purification and concentration in an Amicon cell. SDS−PAGE protein profiles of fractions are shown in Figure 2. [b] BDH activity was measured at 30 °C after preincubation with PC/PE/DPG vesicles in the cuvette (see Experimental Procedures). [c] The DEAE fraction has a specific activity of 130 ± 12 at 37 °C.

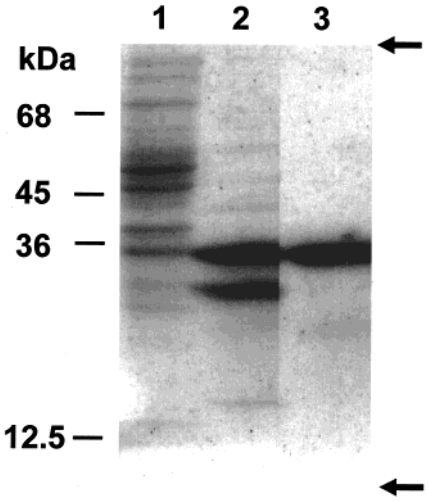

FIGURE 2: Gel electrophoresis of fractions from purification of HH-Histag-BDH. Protein samples from each step (see Table 1) were separated by SDS−PAGE and stained with Coomassie blue. Lane 1 (~20 μg of protein) is the lysate, lane 2 (10 μg of protein) is the fraction after the His-Trap column, and lane 3 (10 μg of protein) is after the DEAE column and concentration. The arrows denote the top of the resolving gel and the dye front.

N-terminal Histag peptide was cleaved from the purified HH-Histag-BDH fusion protein by treatment with enterokinase as detected by SDS−PAGE (Figure 3). Cleavage is >90% complete after 1 h (as determined by densitometry) and complete after 2 h (not shown), yielding a cleavage product with a molecular mass of 32.5 kDa, similar to that for the mature form of recombinant HH-BDH expressed in Sf9 insect cells (*9*). In this gel system (see Figure 3), HH-BDH (lane 2) appears to migrate slightly faster than BH-BDH (lane 3), but this difference is not significant. The presumed second product of the enterokinase cleavage (i.e., the 17-residue peptide from the N-terminus of HH-Histag-BDH) was not detected using this SDS−PAGE system. Enterokinase treatment does not alter the size of BH-BDH (Figure 3, lanes 3 and 4), and neither is recombinant HH-BDH (expressed in insect cells) susceptible to digestion by enterokinase (data not shown). Treatment of HH-Histag-BDH with enterokinase for 2 h (to obtain complete cleavage of the fusion protein)

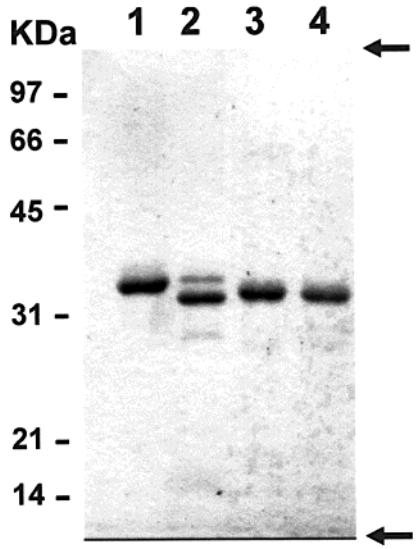

FIGURE 3: Enterokinase cleavage of HH-Histag-BDH. HH-Histag-BDH or BH-BDH was incubated with enterokinase for 1 h at room temperature (see Experimental Procedures), and aliquots (0.75 μg of protein) of each were analyzed by SDS−PAGE with Coomassie Blue staining. Lanes 1 and 2 are HH-Histag-BDH before and after digestion, respectively. Lanes 3 and 4 are controls with BH-BDH before and after incubation with enterokinase, respectively. The apparent difference in migration of HH-BDH (lane 2) and BH-BDH (lanes 3 and 4) is not significant. The arrows denote the top of the resolving gel and the dye front.

did not significantly change the BDH specific activity as compared with controls (up to ~20% loss of activity in the absence of enterokinase). Thus, catalysis by HH-Histag-BDH is unaffected by the 17 additional residues fused to the N-terminus.

The enzymic activity of HH-Histag-BDH, both in the *E. coli* lysate and throughout the purification, was manifest only after reconstitution with phospholipid vesicles containing PC (not shown). The purified HH-Histag-BDH is activated by lipid vesicles that contain PC, e.g., prepared from either PC alone or the ternary mixture of PC with PE and DPG. For both types of lipid vesicles, the activation characteristics are comparable to those for BH-BDH (Figure 4). As for BH-BDH, maximum activation of recombinant HH-Histag-BDH was obtained with PC/PE/DPG vesicles, a lipid mixture that mimics the mixture of mitochondrial lipids (*41*). For each of the three enzymes reactivated with this mixture, the efficiency of activation, i.e., the molar ratio of PC to BDH to effect 50% reactivation, is comparable (ranging from 7.5 to 11.5 mol of PC/mol of BDH; see legend to Figure 4). As described previously for BH-BDH (see ref *41*), a single molecular species of PC (i.e., dioleoyl-PC) is sufficient to restore essentially full activity (>80%) to either the natural or recombinant proteins albeit with reduced efficiency as compared with that afforded by the PC/PE/DPG vesicles. Notably, with the PC vesicles, the efficiency of reactivation of the recombinant proteins, HH-Histag-BDH and the C69A/C242A Histag-BDH mutant (23 and 28 mol of PC/mol of BDH monomer, respectively), is somewhat lower (more efficient) than is found for PC activation of BH-BDH (half-maximal activation at 39 mol of PC/mol of BH-BDH; see legend to Figure 4). As with BH-BDH, lipid vesicles devoid of PC, e.g., PE/DPG, do not activate HH-Histag-BDH (not shown). Thus, the purified recombinant HH-BDH fusion protein, HH-Histag-BDH, exhibits the specific PC require-

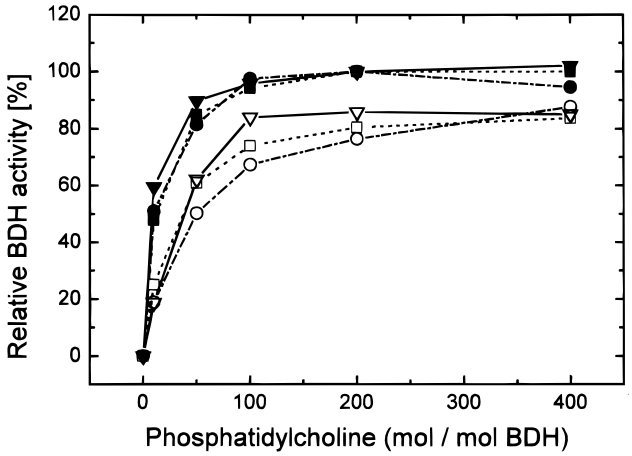

FIGURE 4: Activation of HH-Histag-BDH (■, □), the C69A/C242A mutant (▼, ▽), and BH-BDH (●, ○) by phospholipid vesicles. Each BDH preparation was preincubated with various amounts (as indicated) of phospholipid vesicles composed of either PC alone (open symbols) or PC/PE/DPG (5:4:1 molar ratio, closed symbols) prior to the measurement (see Experimental Procedures) of BDH activity at 37 °C (expressed as the percentage with respect to the maximal activation with PC/PE/DPG, 130 μmol of NAD^+ reduced·min^{-1}·mg^{-1}). The efficiencies of reactivation (the mole ratio of PC to BDH to obtain 50% reactivation) for the BH-BDH, HH-Histag-BDH, and C69A/C242A mutant were 10, 11.5, and 7.5 (with PC/PE/DPG vesicles) and 39, 23, and 28 (with PC vesicles), respectively.

ment for function that is a defining characteristic of BDH. The measured kinetic parameters for the purified HH-Histag-BDH fusion protein and for HH-BDH (obtained after complete cleavage with enterokinase) are similar to each other and to those reported previously for both BH-BDH and recombinant HH-BDH, expressed in Sf9 insect cells (see Table 2). The results show that both recombinant forms of BDH, HH-Histag-BDH and HH-BDH derived therefrom, each require PC for function and have enzymic properties comparable to those of purified BH-BDH.

A structural model for HH-BDH (Figure 5) was developed by folding its primary sequence according to the coordinates of the homologous 3α,20β-hydroxysteroid dehydrogenase (*19*), followed by energy minimization using the programs QUANTA and CHARMm (see ref *42*). The N-terminal domain as well as the central domain (i.e., residues 1−193 of HH-BDH) yielded a structure with high confidence (red and green in Figure 5) that exhibits features similar to those of the homologous SC-ADH (consistent with high homology of these domains of HH-BDH to the SC-ADH). However, due to lack of sequence homology in the C-terminal domain (residues 194−297 of HH-BDH), a reliable structure prediction was not achieved for this segment as evidenced by a lack of any identifiable secondary structure in this domain. In the predicted model (Figure 5), four cysteines (residues 17, 40, 163, and 175) of HH-BDH appear as pairs (not shown), consistent with the formation of two disulfide bonds as identified in BH-BDH (*18*). C69 appears unpaired and near the adenosine moiety of NAD (the position of which is well defined by the structure of the conserved SC-ADH domain of BDH). The rapidly reacting sulfhydryl (SH1) of BH-BDH (*43*) was found to correspond in HH-BDH to C242 (*6*), the sixth cysteine of the enzyme. Although the predicted structure of the C-terminal domain of BDH is not as well defined as the conserved domains of this SC-ADH (see Experimental Procedures), the energy-minimized model (Figure 5) places C242 (the only cysteine in this domain) in the vicinity of the nicotinamide moiety of NAD and presumably proximal to the substrate. While the specific position of C242 remains to be confirmed, its predicted location in the structural model is not inconsistent with the prior chemical modification studies (see Discussion). The model identifies C69 and C242 as the two sulfhydryls, defines the location of C69, and indicates that both sulfhydryls are near the active site of the enzyme as had been predicted (*23*, *24*).

Six single-site or double mutants of HH-Histag-BDH were prepared that lacked either one or both of the sulfhydryls, C69 and C242, substituting each with either A or S. The yields and purity of the different mutant enzyme preparations (four individual mutations, C69A, C69S, C242A, and C242S, and two double mutations, C69A/C242A and C69A/C242S) were each found to be similar (not shown) to those for the unmodified recombinant HH-Histag-BDH. The lower specific activity of the C69S mutant as compared with the other proteins (see Table 2, footnote *a*) may not necessarily reflect an intrinsically lower catalytic turnover rate for this enzyme since the preparation of this mutant was not optimized. With the corresponding C69A mutant, initial preparations also had lower specific activities (similar to those of C69S) but with otherwise similar kinetic parameters (see Table 2, footnote *c*). In this regard, it is notable that, except for their different specific activities, the C69A and C69S have essentially identical kinetic parameters (see below) that are comparable to the unmodified HH-Histag-BDH. As purified, each mutant is inactive and requires reconstitution with lipid vesicles containing PC before efficient catalysis is manifest (not shown). All six mutants of HH-Histag-BDH are active after reconstitution with PC (see Table 2, footnote *a*) so that neither of the two cysteines, C69 and C242, is essential for catalysis. For the C69A/C242A double mutant, the PC-dependent activation profiles (by either PC alone or PC/PE/DPG vesicles) are comparable to the corresponding activation profiles for the unmodified HH-Histag-BDH (Figure 4), demonstrating that neither of these cysteines is required for the PC-dependent activation of the enzyme.

Purified bovine heart BDH is susceptible to aerobic inactivation in the absence of a reducing agent such as DTT (*22*). In the absence of DTT, HH-Histag-BDH also becomes inactivated with a half-time of ∼10−20 h (Figure 6); the addition of DTT protects the enzyme for a prolonged period (up to at least 72 h) against aerobic inactivation. The double cysteine mutant, C69A/C242A, is stable in the presence or absence of DTT (Figure 6), demonstrating that the inactivation of HH-Histag-BDH is referable to one or both of these two cysteines. The two classes of single cysteine mutants (at C69 versus C242) exhibit different aerobic stability properties. The C242A and C242S mutants are stable with and without DTT (not shown) and are thus comparable to the C69A/C242A mutant. On the other hand, the C69A mutant (Figure 6) (or C69S, not shown) behaves like HH-Histag-BDH and becomes inactivated in the absence of DTT, losing 70−80% of its initial activity within 24 h. In the presence of DTT, C69A is stable for about 24 h but then becomes inactivated, presumably due to depletion of the DTT and subsequent oxidation of C242. The C69A mutant but not the C242A mutant was also found to be inactivated (to

Table 2: Function of Recombinant Human BDH and Cysteine Mutants[a]

BDH sample	K_m^{HOB} (mM)	K_m^{NAD} (mM)	K_m^{AcAc} (mM)	K_m^{NADH} (mM)
recombinant BDH				
HH-Histag-BDH	0.64 ± 0.09	0.41 ± 0.05	0.10 ± 0.03	0.013 ± 0.004
HH-BDH[b]	0.24 ± 0.11	0.74 ± 0.15	0.19 ± 0.03	0.012 ± 0.002
HH-Histag-BDH mutants				
C69A[c]	1.0 ± 0.2	0.54 ± 0.06	0.22 ± 0.02	0.044 ± 0.023
C69S	1.3 ± 0.1	0.35 ± 0.08	0.43 ± 0.14	0.035 ± 0.010
C242A	0.85 ± 0.12	0.38 ± 0.06	0.33 ± 0.10	0.026 ± 0.010
C242S	6.2 ± 2.0	0.43 ± 0.10	0.70 ± 0.09	0.039 ± 0.010
C69A/C242A	1.5 ± 0.1	0.36 ± 0.07	0.40 ± 0.11	0.077 ± 0.013
C69A/C242S	6.7 ± 0.9	0.50 ± 0.04	1.26 ± 0.25	0.06 ± 0.02
literature values				
BH-BDH	0.50 ± 0.11[d]	0.95 ± 0.15[d]	0.50[e]	0.014[e]
HH-BDH (Sf9 cells)	0.71 ± 0.11[d]	0.53 ± 0.08[d]		

[a] Enzymatic activities of HH-Histag-BDH, HH-BDH, and the single or double C69 and C242 mutants of HH-Histag-BDH (each reconstituted with PC/PE/DPG vesicles) were measured at 30 °C as a function of (*R*)-HOB, NAD^+, AcAc, or NADH to determine the apparent Michaelis constants (K_m) for the substrates and coenzymes (see Experimental Procedures for details). The K_m^{HOB} was determined at 20 mM NAD^+ with 0.5−10 mM (*R*)-HOB (for C242S and C69A/C242S, (*R*)-HOB was varied from 0.5 to 50 mM); $K_m^{NAD^+}$ was determined at 10 mM (*R*)-HOB with 0.1−20 mM NAD^+; K_m^{AcAc} was determined at 0.2 mM NADH with 0.01−5 mM AcAc; K_m^{NADH} was determined at 5 mM AcAc with 0.005−0.2 mM NADH. The apparent V_{max} values calculated from the K_m^{HOB} and $K_m^{NAD^+}$ data (at 30 °C) were averaged, and the values (μmol of NAD^+ reduced·min^{-1}·mg^{-1} ± SEM) are given in parentheses following each enzyme: HH-Histag-BDH (65 ± 6), HH-BDH (49 ± 6), C69A (61 ± 6), C69S (28 ± 4), C242A (64 ± 5), C242S (37 ± 3), C69A/C242A (66 ± 6), and C69A/C242S (71 ± 6). The values shown are typical of two or more independent preparations of each enzyme. [b] HH-BDH was prepared by enterokinase treatment of HH-Histag-BDH (see Figure 3). [c] Initial preparations of the C69A mutant had lower specific activities (20−30 μmol of NAD^+ reduced min^{-1}·mg^{-1} at 30 °C) but with otherwise similar kinetic parameters. [d] Reference *9*. [e] Reference *44*; see also ref *48*.

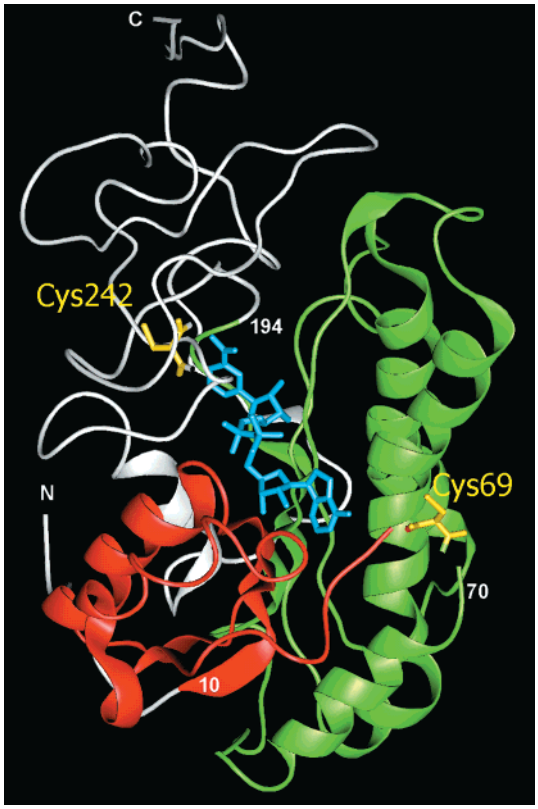

FIGURE 5: Computed model of the HH-BDH structure. The 3-D structure of a single protomer of HH-BDH was modeled and energy minimized according to the coordinates of the homologous 3α,-20β-hydroxysteroid dehydrogenase using the programs QUANTA and CHARMm. In the lipid binding domain (white), there is little homology between the two sequences, and therefore the structure in this region is poorly constrained. Color code: red, NAD(H) binding domain (residues 10−69); green, substrate binding domain (residues 70−193); white, lipid binding domain (residues 194−297) and N-terminal tail (residues 1−9); yellow, cysteines 69 and 242; blue, NAD(H).

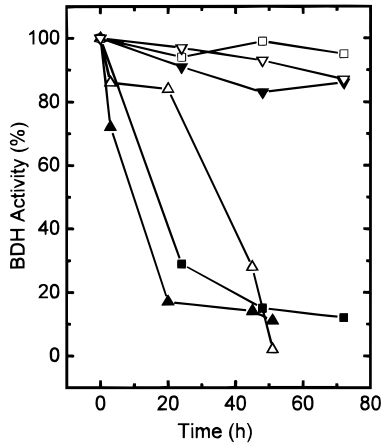

FIGURE 6: Stability of the HH-Histag-BDH and cysteine mutants in the presence and absence of DTT. Each BDH preparation was reconstituted with PC vesicles (200 mol/mol of BDH) in 20 mM HEPES−NaOH and 1 mM EDTA (pH 7.0), incubated at 4 °C in either the absence (filled symbols) or presence (open symbols) of 10 mM DTT, and assayed for BDH activity at the times indicated. Data are expressed as the percentage with respect to the initial activity of each sample. Key: HH-Histag-BDH (■, □), C69A mutant (▲, △), and C69A/C242A mutant (▼, ▽).

<1%) by treatment with sulfhydryl-specific reagents, such as methanethiosulfonate or *N*-ethylmaleimide (De and Trommer, unpublished results). In the stability studies, analysis of each of the enzyme and mutant samples by SDS−PAGE (with either Coomassie or silver staining) did not indicate any significant proteolysis within the 72 h of the study (not shown). The aerobic stability of the C242A and C242S mutants together with the inactivation of the C69A mutant by sulfhydryl reagents is consistent with C242 being the reactive sulfhydryl of BDH (i.e., SH1; see Discussion). From these studies, it is concluded that the sulfhydryl of C242 confers to BDH its susceptibility to aerobic inactivation.

The enzyme kinetic parameters of the six different cysteine mutants were measured and compared with those for HH-Histag-BDH (Table 2). For all six mutants, the apparent K_m values for NAD^+ are comparable and similar to those for unmodified HH-Histag-BDH that has both of its sulfhydryls. In the reverse assay, the K_m^{NADH} is increased ~2−3-fold for

the single mutants and ~5-fold for the double mutants as compared with HH-Histag-BDH. Each mutant exhibits increased K_m values for substrates. For the C69A, C69S, C242A, and C69A/C242A enzymes, the K_m^{HOB} and K_m^{AcAc} are similar or only slightly higher (up to ~2.5-fold) than those of the unmodified enzyme. However, the C242S and C69A/C242S enzymes each exhibit significantly higher K_m values for the substrates (Table 2); for these mutants with serine substituted for C242, the K_m^{HOB} and K_m^{AcAc} values are each increased ~7−12-fold compared with those for HH-Histag-BDH.

DISCUSSION

The expression and purification of the full-length mature form of HH-BDH has been achieved via the design and construction of a plasmid, pBDH-VU1, that provides IPTG-inducible expression of a novel HH-BDH fusion protein, HH-Histag-BDH. With this new expression system, HH-Histag-BDH is produced in the cytosol of *E. coli* from which it has been purified and characterized. HH-Histag-BDH consists of the complete sequence of the mature form of HH-BDH fused at its N-terminus with an enterokinase-cleavable hexahistidine affinity tag. The purified fusion protein is inactive in the absence of lipid and exhibits the specific requirement of PC for function that is characteristic of BDH; i.e., efficient catalysis by HH-Histag-BDH requires its prior reconstitution with phospholipid vesicles containing PC. By site-directed mutagenesis of this enzyme, putative functional roles of C69 and C242, two of the six cysteines in HH-BDH, have been investigated. A set of six mutants, in which these two cysteines (predicted to constitute the two active center sulfhydryls of BDH; see below) have been replaced with either alanine or serine, has been constructed, purified, and characterized. In this set of mutants, the largest change in enzymic function is an ~10-fold increase in the apparent K_ms for the substrates (K_m^{HOB} and K_m^{AcAc}) that is found for both the C242S and C69A/C242S mutants (Table 2) in which the SH at C242 is replaced by OH. Although BDH mutants lacking either or both C69 and C242 exhibit changes of up to an order of magnitude in kinetic parameters, each of these mutant enzymes requires PC for function and is catalytically active. Further, the two double mutants (C69A/C242A and C69A/C242S), each lacking both sulfhydryls, are also active, demonstrating, for the first time, that catalysis by BDH does not require either of these cysteines.

BDH is a ubiquitous mitochondrial enzyme (*45*, *46*) that is located on the inner face of the mitochondrial inner membrane (*47*). In mammals, this enzyme serves to interconvert the two major ketone bodies [(*R*)-HOB and AcAc] and is therefore essential for their normal metabolism. The first purification of BDH approaching homogeneity was from bovine heart mitochondria and took about 60 h (*4*), which was subsequently reduced to ~24 h (*3*). By contrast, the purification of HH-Histag-BDH is accomplished in ~7 h (starting from frozen pellets of *E. coli*), about 3-fold faster than can be achieved for the mitochondrial enzyme. The cleavable hexahistidine affinity tag at the N-terminus of HH-Histag-BDH provides for rapid purification of the enzyme via a two-step sequential affinity and ion-exchange chromatography method that yields purified HH-Histag-BDH with specific activity comparable to that of BDH purified from either bovine heart or rat liver (*3*). The power of the new expression and purification method described here is that mutants of the full-length HH-BDH protein can readily be prepared, expressed, and purified following a similar protocol as is demonstrated with the six cysteine mutants of BDH (see Table 2).

BDH specifically requires PC for enzymic function, and for the natural enzyme, mitochondrial PC fulfills the lipid requirement. In previous studies of recombinant HH-BDH, the full-length mature form of the enzyme, i.e., lacking its mitochondrial targeting leader sequence (*8*), was expressed in a catalytically active form in Sf9 cells infected with BDH− cDNA in baculovirus (*9*). In Sf9 cells, recombinant HH-BDH was constitutively active (its lipid requirement being fulfilled by the endogenous PC present in the cellular membranes) with enzyme kinetic parameters (*9*) similar to those for BH-BDH in mitochondria (*44*, *48*). Both HH-BDH (*9*) and recombinant forms of rat liver BDH (*18*, *19*) exhibit a PC requirement for function. As expressed in the *E. coli* cytosol, the recombinant human enzyme, HH-Histag-BDH, is inactive but can be reactivated in the bacterial lysate by reconstitution with PC vesicles, consistent with a PC requirement for catalysis. Purified HH-Histag-BDH exhibits PC-dependent activation characteristics comparable to those for BH-BDH (see Figure 4). As observed previously with BH-BDH (*15*, *41*), maximal activation of HH-Histag-BDH was obtained with PC/PE/DPG lipid vesicles that mimic the natural phospholipid mixture of mitochondria (i.e., mitochondrial phospholipid). With this lipid mixture, the efficiency of activation is similar for each enzyme (BH-BDH, HH-Histag-BDH, and the C69A/C242A mutant). However, dioleoyl-PC alone is sufficient to activate both HH-Histag-BDH and the C69A/C242A mutant to a specific activity comparable to that observed with the BH enzyme (~85% of that obtained with PC/PE/DPG vesicles). The somewhat lower maximal activation of BDH by PC versus PC/PE/DPG vesicles is a well-established characteristic of the PC-dependent activation of the enzyme (*4*, *41*) that may be related to subtle changes in enzyme kinetic parameters observed with BDH in the presence of negatively charged lipids such as DPG (*41*, *44*, *48*). By contrast with the similar activation efficiencies found with PC/PE/DPG vesicles, HH-Histag-BDH activation by vesicles of PC alone is more efficient (the efficiency of activation is ~2-fold lower; see Figure 4 legend) than is that for BH-BDH, which suggests that the Histag (with its charged residues) may facilitate interaction of the protein with PC vesicles. In addition to its unremarkable PC-dependent activation characteristics, the measured enzyme kinetic parameters of HH-Histag-BDH are found to be similar to those for the different species of BDH as well as for HH-BDH in Sf9 cells (Table 2). Thus, this new recombinant form of the enzyme has normal catalytic function.

HH-Histag-BDH was designed with an enterokinase cleavage site adjacent to the natural N-terminus of the mature mitochondrial form of HH-BDH. Enterokinase cleavage of purified HH-Histag-BDH yields the mature form of HH-BDH (see Figure 3) without any significant change in catalytic activity. After complete cleavage to remove the Histag, the measured enzyme kinetic parameters of HH-BDH were found to be similar to those of the original uncleaved HH-Histag-BDH (see Table 2). Although a decrease (up to 20%) in enzymic activity was observed in some enterokinase

cleavage experiments, such losses were comparable to those in controls without enterokinase. Thus, the N-terminal linked 17-residue peptide of HH-Histag-BDH does not affect catalysis by this enzyme. Previous studies of recombinant rat liver BDH had indicated that the N-terminus of the protein is not critical for catalysis since this enzyme exhibits activity either as a galactosidase fusion protein or when lacking five N-terminal residues of the mature enzyme (*25*, *26*). Our finding that HH-Histag-BDH is fully active after reconstitution with PC confirms our previous finding that the N-terminal mitochondrial targeting leader sequence of precursor BDH is not required for expression of the functional enzyme, as had been demonstrated by expression, in Sf9 cells, of functional HH-BDH lacking this segment (*9*).

Chemical derivatization studies had implicated the cysteines of BDH in its enzymic function (*21*, *22*, *49*). However, cysteine residues are not conserved in the SC-ADH, being often absent from the prokaryotic SC-ADH though present in eukaryotic SC-ADH [ranging up to as many as 14 cysteines in one of the steroid dehydrogenases (*50*)]. As corrected (see Experimental Procedures), the sequence of HH-BDH has a total of six cysteines that are identical to those in the rat liver and BH enzymes (*6*, *7*). This cysteine conservation is consistent with a postulated role of these residues in the structure and/or function of BDH although it may also reflect the high interspecies homology of the protein (see ref *9*). In BH-BDH, two of the cysteines are in the reduced sulfhydryl form (SH1 and SH2) with the other four forming disulfides (*18*). The two cysteines of BDH, C69 and C242, were selected for mutagenesis on the basis of the prediction from the structural model that neither of these residues participates in the two disulfides present per protomer of BDH**,** with C69 being identified as SH2 and located near the adenosine moiety of NAD (Figure 5). A peptide that included the rapidly reacting cysteine (*43*) provided the identification of SH1 as C242 of HH-BDH (*6*). Hatefi's group recognized that the peptide containing the reactive sulfhydryl might represent a part of the nicotinamide rather than the adenosine binding site of BDH (*43*). Indeed, the model (Figure 5) predicts C242 to be near the nicotinamide moiety of NAD (i.e., the catalytic site), a location concordant with chemical modification studies; e.g., modification of C242 by cyanylation, a relatively small change, alters catalytic function, increasing the K_m^{BOH} by ~10-fold (*23*). Its derivatization by an arylazido derivative of NAD indicates that at least part of the C-terminal domain, including C242, is folded over the active center of the enzyme (*6*). The structural model of HH-BDH locates the two sulfhydryls (C69 and C242) at either end of the NAD binding site.

Mitochondrial BDH is known to require a reducing environment for stability (*21*, *22*). The inactivation of BDH in the absence of a protective reducing agent such as DTT, as found previously for BH-BDH (*22*), is also observed with HH-Histag-BDH (Figure 6). This instability appears to derive from aerobic oxidation of the sulfhydryl at C242 since mutants lacking this cysteine (e.g., C242A and C69A/C242A) have prolonged stability in the absence of DTT whereas unmodified HH-Histag-BDH and the C69A mutant each are inactivated in a DTT-protectable manner (Figure 6). Likewise, both the C69A and C242A mutants can be derivatized by methanethiosulfonate or *N*-ethylmaleimide (presumably at C242 and C69, respectively), but only the C69A mutant (and not the C242A mutant) is inactivated by such modification (De and Trommer, unpublished results), confirming proximity of C242 to the active center of HH-BDH as in BH-BDH (*23*, *24*).

Contrary to predictions from initial NEM inactivation studies of BDH (*21*, *22*, *49*), more recent chemical derivatization studies had suggested that neither of the individual sulfhydryls, SH1 or SH2, is required for catalysis but rather that the extent of inhibition is dependent on the size of the modifying reagent (*23*, *24*). Chemical modification of SH1 (C242) of BDH has a more profound effect on catalysis (with reduced activity and an increased K_m for substrate) than does derivatization of SH2 (C69). However, the very low or undetectable activity of double derivatives with both sulfhydryls modified (*21*−*24*) suggested the possibility that efficient catalysis by BDH may require the presence of at least one sulfhydryl near the catalytic center. The site-directed mutagenesis studies described here refute this hypothesis, laying to rest the concept that either sulfhydryl is required for efficient catalysis and opening a fresh perspective on the previous sulfhydryl modification studies of BDH (*21*−*24*, *43*, *49*). Taken together, the chemical modification and site-directed mutagenesis studies of BDH are analogous to similar studies of *lac* permease (*51*, *52*). Although classical chemical modification of that transporter had revealed cysteinyl residues at or near the substrate binding site and a cysteine essential for transport (*51*), a *lac* permease, prepared by site-directed mutagenesis to be devoid of cysteine, was fully functional, precluding any role for sulfhydryl in the transport mechanism (*52*). In the studies described here, each of the six HH-BDH mutants, with conservative substitutions of alanine or serine for either one or both of these cysteines (C69 or C242), is active (Table 2), with the C69A, C242A, and C69A/C242A mutants having specific activities similar to that of unmodified HH-BDH. The lower specific activity of the C69S mutant could indicate an intrinsically low enzymatic turnover rate for this modified enzyme since it is similar to the 2-fold reduction in activity (with unchanged K_m^{NAD} or K_m^{BOH}) afforded by *N*-ethylmaleimide derivatization of this sulfhydryl in bovine heart BDH (*24*). However, as its other kinetic parameters were comparable to the analogous C69A mutant, the low activity of C69S may, more likely, be a trivial result, e.g., such as being referable to oxidation of this mutant during its expression and/or purification. The somewhat lower activity of the C242S enzyme preparation may likewise reflect less than optimal expression and/or purification since the C69A/C242S double mutant has a specific activity similar to those of the C69A and C69A/C242A mutants (Table 2, footnote *a*). While two of the single-site mutants have somewhat reduced specific activities, it is clear that the substitution of both C69 and C242 of BDH by either alanine or serine has a more limited effect on the enzymic activity and kinetic parameters (see Table 2) than is afforded by chemical derivatization of both of its sulfhydryls (*21*−*24*). The inactivation of BDH concomitant with sulfhydryl derivatization (*21*−*24*) must therefore derive from perturbation of the catalytic site by the adducts. Site-directed HH-BDH mutants lacking both C69 and C242 are now shown to be highly active, demonstrating unequivocally that neither of these cysteines is required for efficient catalysis by BDH. These single and double cysteine mutants of BDH make possible site-directed spin and

fluorescence labeling for spectroscopic studies that have been initiated to further test predictions from the structural model (Figure 5), particularly the proximity of C242 with respect to the active site.

Each of the set of six cysteine mutants (including the sulfhydryl-free double mutants) of HH-Histag-BDH have kinetic parameters (particularly $K_m^{NAD^+}$ values) that are generally similar to those of the unmodified enzyme (Table 2). Differences include a lower specific activity of some mutants (discussed above) and an increase in the apparent K_m^{NADH} values for each mutant, especially for the C69A/C242A and C69A/C242S double mutants, both of which have an ~5-fold increased K_m^{NADH}. BDH has an ordered sequential reaction mechanism that requires NAD(H) to bind before the substrate such that the catalytic $K_m^{NAD(H)}$ is analogous to the dissociation constant for these ligands (*48*). The observed increases in the apparent K_m^{NADH} for the C69 and C242 mutants (but not in their apparent $K_m^{NAD^+}$) suggest that each of these mutants has subtle changes in the structure of the catalytic center that affect the binding of NADH but not of NAD^+. Thus, the absence of either or both sulfhydryls, C69 and C242, appears to affect the NADH but not the NAD^+ conformation of the enzyme. A more marked and notable difference is that the two C242S mutants (C242S and C69A/C242S) exhibit ~10-fold increases in apparent K_m values for both the oxidizable and reducible substrates [(*R*)-HOB and AcAc]. The increase in K_m^{HOB} for these purified C242S mutants of HH-Histag-BDH is similar to that found previously for the C242S mutant of HH-BDH, as expressed in Sf9 cells (*9*). Both of the C242S mutants of HH-Histag-BDH (C242S and C69A/C242S) exhibit no change in the $K_m^{NAD^+}$ value as compared to the unmodified enzyme and have K_m^{NADH} values not dissimilar from the other mutants. The ~10-fold increases in the substrate K_ms found for the C242S and C69A/C242S mutants suggest that these mutant enzymes each have an altered substrate binding site but little change in their NAD(H) binding site. This increase in substrate K_ms for both C242S mutants is remarkably similar to the effect of single-site cyanylation of BH-BDH [an ~10-fold increase in K_m^{HOB} and K_m^{AcAc} (*18*)]. The altered kinetic parameters of the C242 derivatized enzyme (*23*, *24*) or the C242S mutant expressed in Sf9 cells (*9*) (as now confirmed in this study with the two purified C242S mutants) had suggested a possible role of the C242 sulfhydryl in binding the substrate(s). By contrast, the alanine mutants of C242 (C242A and C69A/C242A) are now found to have unremarkable kinetic parameters with only minor changes in apparent K_m values for the substrates (see Table 2), refuting the postulate that this sulfhydryl directly participates in substrate binding (analogous to its unimportance in catalysis, as discussed above). While replacing the SH at residue 242 with a proton (in the alanine mutants) does not affect the kinetic parameters of the enzyme, its substitution with an OH (as in the serine mutants) significantly reduces the apparent affinity for the substrates (increased K_m), consistent with C242 being in the vicinity of the substrate binding region of the active center as was postulated (*9*) and consistent with the predicted structural model (Figure 5). Thus, changing the SH of C242 to OH or chemically modifying it with even relatively small adducts profoundly changes the affinity of the substrate binding site. For the serine mutants of C242 (i.e., C242S and C69A/C242S), the increase in substrate K_ms can be attributed to a structural perturbation in the substrate binding site (due to the presence of the OH versus the SH at the catalytic center) rather than from the absence of the sulfhydryl, per se. The differences in catalytic properties between the serine versus alanine mutants at C242 of BDH suggest that the $-CH_2SH$ is better mimicked by the methyl of alanine rather than the $-CH_2$-OH of serine. These results are consistent with the finding that, in proteins, free cysteines have more contact to nonpolar residues and are more buried than are the half-cysteines (*53*), i.e., a tendency of cysteine and hydrophobic residues to be in mutual vicinity within the protein core (*54*). By contrast, serine residues in proteins are truly hydrophilic and tend to be located at the protein surface (*55*). Thus, the C242 residue of BDH appears to be in a relatively hydrophobic environment such that the introduction of the more hydrophilic serine at this location yields a protein with a conformation that has reduced affinity for the substrates. Since both BOH and AcAc are polar molecules, the perturbation of substrate binding found with the C242S mutants but not the C242A mutants shows that this residue is not directly involved in binding the hydrophilic substrates (BOH or AcAc). Rather, the replacement of $-SH$ at this residue by $-OH$ would appear to affect catalytic function via some indirect perturbation of the structure of the substrate binding site. Thus, although C242 (located in the C-terminal lipid binding domain of the enzyme) is in the vicinity of the NAD binding site [formed by the N-terminal domains of the protein (*6*, *9*)], this residue would appear to be located in a relatively hydrophobic environment such that it participates only indirectly in defining the substrate binding site at the catalytic center of BDH.

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