Chemical Modification and Site-Directed Mutagenesis Studies of Rat 3-Hydroxyisobutyrate Dehydrogenase†

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ABSTRACT: Rat 3-hydroxyisobutyrate dehydrogenase shares sequence homology with the short-chain alcohol dehydrogenases. Site-directed mutagenesis and chemical modifications were used to examine the roles of cysteine residues and other residues conserved in this family of enzymes. It was found that a highly conserved tyrosine residue, Y162 in 3-hydroxyisobutyrate dehydrogenase, does not function catalytically as it may in other short-chain alcohol dehydrogenases. Of the six cysteine residues present in 3-hydroxyisobutyrate dehydrogenase, only cysteine 215 was found to be critical to catalysis. C215A and C215D mutant enzymes were catalytically inactive but produced CD spectra identical to wild-type enzyme. C215S mutant enzyme displayed a lowered V_{max} than wild-type enzyme, but K_m values were similar to those of wild-type enzyme. The C215S mutant enzyme was inactivated by treatment with phenylmethanesulfonyl fluoride but was not inactivated by treatment with iodoacetate, whereas the wild-type enzyme was inactivated by treatment with iodoacetate but not inactivated by treatment with phenylmethanesulfonyl fluoride. The present data suggest that 3-hydroxyisobutyrate dehydrogenase differs in mechanism from other short-chain alcohol dehydrogenases studied to date and that cysteine 215 has a critical function in catalysis, possibly as a general base catalyst.

(*S*)-3-Hydroxyisobutyrate (HIBA),[1] a central metabolite in the valine catabolic pathway, is reversibly oxidized to methylmalonate semialdehyde by a specific NAD^+-dependent dehydrogenase (HIBADH or 3-hydroxy-2-methylpropanoate:NAD^+ oxidoreductase, EC 1.1.1.31) present in mitochondria (Robinson et al., 1957). The metabolism of HIBA is of interest because it lacks the CoA moiety of most intermediates in this pathway and thus can be released into the blood stream by specific tissues. It is cleared by the liver and serves as a good substrate for hepatic gluconeogenesis (Letto et al., 1986). Elevated levels of HIBA in serum and urine have been reported for a number of disease states (Congdon et al., 1981; Landaas et al., 1975).

Amino acid sequence comparisons of rat liver HIBADH with other dehydrogenases revealed significant homology with the short-chain alcohol dehydrogenases, a diverse family of enzymes believed to be structurally, and perhaps evolutionarily, related (Crabb et al., 1991). All known members of this family of enzymes contain a conserved tyrosine residue which in some cases has been shown through site-directed mutagenesis to be critical for catalysis (Albalat et al., 1992; Chen et al., 1993; Ensor et al., 1991). Chen et al. (1993) have proposed that this residue in *Drosophila* alcohol dehydrogenase (ADH) acts as a catalytic base which abstracts the alcohol proton and that surrounding residues, possibly a highly conserved lysine, may facilitate electron transfer by lowering the pK_a of the phenolic side chain. In contrast to this mechanism, studies of 3α-hydroxysteroid/dihydrodiol dehydrogenase (Pawlowski et al., 1994) demonstrated that the corresponding conserved tyrosine residue does not function catalytically in this enzyme and suggested that another residue, not conserved among short-chain alcohol dehydrogenases, may act as a general base catalyst responsible for proton abstraction from the steroid substrate. All of the most highly conserved residues characteristic of the short-chain alcohol dehydrogenases are present in rat HIBADH with the exception that the highly conserved lysine residue (K157 of *Drosophila* ADH and K155 in 15-hydroxyprostaglandin dehydrogenase) is a valine in HIBADH (Crabb et al., 1991). Another salient difference is that HIBADH is very sensitive to thiol-reactive compounds, while some short-chain alcohol dehydrogenases are insensitive or even completely lack cysteine residues. *Drosophila* ADH contains two cysteines and can be completely inactivated by various thiol-reactive compounds, but site-directed mutagenesis studies have shown that neither are crucial for enzyme activity (Chen et al., 1990). Whether specific cysteine residues play a crucial catalytic role in HIBADH has not been previously determined. In the present study, we have used chemical modification and site-directed mutagenesis to examine the problem of whether cysteine residues and other residues conserved among short-chain

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[1] Abbreviations: ADH, alcohol dehydrogenase; BDH, 3-hydroxybutyrate dehydrogenase; CoA, coenzyme A; GST, glutathione *S*-transferase; HIBA, 3-hydroxyisobutyrate; HIBADH, 3-hydroxyisobutyrate dehydrogenase; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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dehydrogenases serve critical functions in catalysis by HIBADH. It is shown that the conserved tyrosine residue (Y162 of rat HIBADH) does not act as a catalytic base as it may in *Drosophila* ADH and that this function may be performed instead by a cysteine residue (C215).

EXPERIMENTAL PROCEDURES

Materials. The oligonucleotide-directed in vitro mutagenesis system and *Escherichia coli* strain TG1 were obtained from Amersham. The Sequenase DNA sequencing kit was obtained from U.S. Biochemicals. The plasmid pBluescript II KS+ and the VCS-M13 helper phage were from Stratagene. All restriction enzymes and DNA-modifying enzymes were obtained from Gibco BRL. (*S*)- and (*R*)-methyl-3-hydroxy-2-methylpropionate were from Aldrich. (*S*)- and (*R*)-3-hydroxyisobutyrate phenyethylamine salts were prepared from the corresponding methyl esters as described previously (Rougraff et al., 1988). Glutathione, glutathione−agarose, Reactive Red 120−agarose, thrombin, and all other chemicals were obtained from Sigma Chemical Co. The plasmid expression vector pGEX-KG (Guan & Dixon, 1990) was a kind gift of Dr. Jack Dixon (University of Michigan, Ann Arbor, MI).

Construction of pGEX-HIBADH Expression Vector. A vector for expression of HIBADH as a glutathione *S*-transferase (GST) fusion protein was constructed as follows. A 962-bp DNA fragment was isolated from the original rat liver cDNA (Rougraff et al., 1989) by digestion with *Xba*I followed by partial digestion with *Dde*I. This fragment contains the entire coding region of the mature HIBADH peptide minus the first three residues of the N-terminus (Ala-Ser-Lys). Two complementary synthetic oligonucleotides, 5′-GATCCGGTGGTGGTGGTCATATGGCTTC-3′ and 5′-TTAGAAGCCATATGACCACCACCACCG-3′, were phosphorylated with T4 polynucleoside kinase and annealed by heating to 65 °C and slowly cooling to 25 °C over 30 min. The annealed oligonucleotides, containing the missing N-terminal codons, were then ligated to the *Dde*I/*Xba*I fragment. The resulting full-length cDNA was purified by agarose gel electrophoresis and ligated to *Bam*HI/*Xba*I-digested, phosphatase-treated vector pGEX-KG, placing the HIBADH sequence in frame with GST and separating them by the "kinker" peptide (Guan & Dixon, 1990) Gly-Gly-Gly-Gly-His-Met. The fidelity and reading frame of this construct was confirmed by dideoxynucleotide DNA sequencing.

Oligonucleotide-Directed Mutagenesis. For subsequent mutagenesis, the *Bam*HI/*Xba*I fragment of the pGEX-HIBADH expression plasmid vector was subcloned into the *Bam*HI and *Xba*I sites of pBluescript II KS+, and single-stranded DNA was isolated according to the manufacturer's protocol (Stratagene). Synthetic oligonucleotides were designed to direct the synthesis of the desired mutations. All mutant DNAs were analyzed by double-stranded DNA sequencing to confirm the fidelity of the mutant sequences. Mutant HIBADH cDNAs were excised from pBluescript using *Bam*HI and *Xba*I nad religated to the *Bam*HI and *Xba*I sites of pGEX-KG. Mutant pGEX-HIBADH expression plasmids were analyzed by restriction analysis and DNA sequencing to insure the correct ligation of the mutant cDNAs. Each pGEX expression vector was analyzed by double-stranded DNA sequencing, and mutant plasmids resulting in inactive enzymes (Y162A, V166K, C215A, and C215D) were sequenced in their entirety to confirm that the lack of enzyme activity was not due to incorrect ligation, frame shifts, or other undesired mutations.

Expression and Purification of Wild-Type and Mutant 3-Hydroxisobutyrate Dehydrogenases. TG1 *E. coli* cells harboring the pGEX-HIBADH plasmid expression vectors were grown at 37 °C in TY media containing 100 μg/mL ampicillin until an optical density at 600 nm of approximately 1.0 was reached. Isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM, and cell growth was continued for 15−18 h. Cells were then pelleted by centrifugation and resuspended in 10 volumes of buffer A (PBS: 138 mM NaCl, 2.7 mM KCl, 1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4 containing 0.5% Triton X-100, 0.2 mM NAD^+, 10 mM 2-mercaptoethanol, and 100 μg/mL each of benzamidine and PMSF). C215S mutant HIBADH was purified in the absence of PMSF. The cells were disrupted by sonification with a Sonifier Cell Disruptor W185 (Branson Sonic Power Co., Plainview, NY). The resulting homogeneates were centrifuged at 20000*g* for 30 min at 4 °C, and the extracts were immediately used to purify the recombinant fusion proteins by glutathione affinity chromatography according to the method of Guan and Dixon (1990). The purified GST-HIBADH fusion peptides were cleaved with thrombin as described (Guan & Dixon, 1990) and filtered through fresh glutathione−agarose to remove free GST. The second glutathione−agarose fractions (flow-through) were applied to 1 mL columns of Reactive Red 120−agarose previously equilibrated in thrombin buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 2.5 mM $CaCl_2$, and 10 mM 2-mercaptoethanol]. The columns were then washed with 25 volumes of thrombin buffer followed by elution with buffer containing 0.5 M NaCl and 10 mM NAD^+. The eluted fractions containing HIBADH were concentrated by ultrafiltration, dialyzed two times at 4 °C against 4 L of buffer A, and stored a 4 °C. The purified enzymes were stable for more than 2 months.

Enzyme Assay and Kinetic Analysis. 3-Hydroxyisobutyrate dehydrogenase activity was assayed at 30 °C in a volume of 1 mL containing 50 mM glycine (pH 10.0), 1 mM NAD^+, 1 mM 2-mercaptoethanol, 1 mM K-EDTA, and 2 mM (*S*)-3-hydroxyisobutyrate. Production of NADH was measured by absorbance at 340 nm in a Beckman model 2400 spectrophotometer. One unit of activity was defined as the production of 1 μmol of NADH/min at 30 °C. For kinetic studies, 3-hydroxyisobutyrate concentrations were varied from 35 to 350 μM for all enzymes except the C163A mutant, in which case the concentrations were varied from 160 μM to 3.2 mM. NAD^+ concentrations were varied from 10 to 250 μM. NADH inhibition was determined as previously described (Rougraff et al., 1988). Kinetic data were analyzed as previously described (Rougraff et al., 1988). The concentrations of 3-hydroxyisobutyrate used were determined by an end point assay method (Rougraff et al., 1990), and the concentrations of NAD^+ and NADH used were determined by absorbance.

Chemical Modification. Aliquots of each enzyme were dialyzed overnight at 4 °C in buffer A minus NAD^+ and 2-mercaptoethanol unless otherwise stated. Enzymes were preincubated with the indicated amounts of each reagent at 25 °C and then immediately assayed for 3-hydroxyisobutyrate dehydrogenase activity.

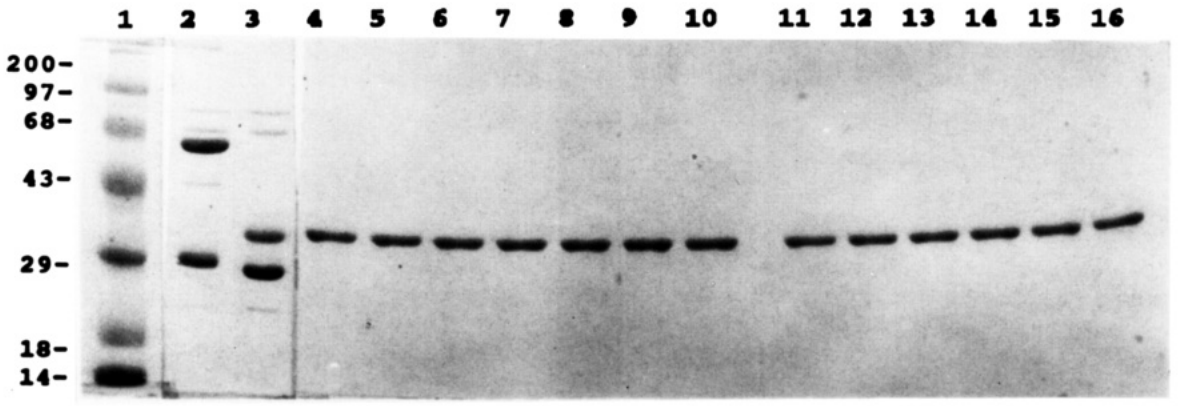

FIGURE 1: SDS−PAGE analysis of purified wild-type and mutant HIBADHs. Lane 1, molecular weight standards, including myosin (H-chain), phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin, and lysozyme; lane 2, 5 μg of wild-type GST-HIBADH; lane 3, 5 μg of wild-type GST-HIBADH after thrombin cleavage; lanes 4−16, 2 μg of purified HIBADH (wild-type, Y162F, Y162A, Y32F, V166K, C39A, C155A, C163A, C175A, C215A, C215D, C215S, and C278A).

CD Spectrapolarimetry. CD spectra were recorded at 21 °C with a Jasco J-720 spectrapolarimeter. Enzyme concentrations were in the range of 80−100 μg/mL with a cell path length of 0.1 cm and a wavelength range of 300−190 nm. Secondary structure contents were estimated using the reference spectra of Yang et al. (1986) and the SSE-338 program (Japan Spectroscopic Co., Tokyo, Japan).

Other Methods. Protein concentrations were determined using the Bradford protein assay (Bradford, 1976) with bovine serum albumin used as standard. SDS−PAGE was performed as described by Laemmli (1970).

RESULTS

Expression and Purification of Wild-Type and Mutant 3-Hydroxyisobutyrate Dehydrogenases. The pGEX expression system proved efficient for the expression of soluble, enzymatically active GST-HIBADH fusion protein. Typically, 10−12 mg of purified fusion protein was obtained per liter of bacterial culture, and all of the mutants produced in this study were expressed to similar extents (Figure 1). Thrombin cleavage was complete in 30 min at room temperature, and no extraneous proteolysis occurred. A second glutathione−agarose chromatography removed most of the free GST peptide; however, this separation was often not complete. Therefore, affinity chromatography with Reactive Red 120−agarose (Ashton & Polya, 1978) was used for final purification (Figure 1), resulting in homogeneous HIBADH with nearly quantitative recovery. Wild-type enzyme purified in this way had specific activity and kinetic parameters (Table 1) very similar to values previously reported for HIBADH purified from rabbit liver (V_{max}, 11.4 units/mg; K_m(HIBA), 61 μM; K_m(NAD^+), 23 μM; Rougraff et al., 1988). K_{ia}(NAD^+) and K_{ib}(HIBA) were also found to be similar to values previously reported for native enzyme (27 and 94 μM, respectively; Rougraff et al., 1988). NADH inhibition studies confirmed that the recombinant enzyme follows the same ordered bi−bi reaction mechanism as previously reported for native HIBADH (Rougraff et al., 1988) and that this binding order was not affected by any of the mutations produced in this study.

Site-Directed Mutants: Conserved Residues. Several studies using site-directed mutagenesis have demonstrated that, for some short-chain dehydrogenases, mutation of a highly conserved tyrosine (Y162 in HIBADH) to phenylalanine resulted in inactive enzymes (Chen et al., 1993; Ensor et al., 1991), suggesting a critical catalytic function for the phenolic side chain. The Y162F mutant HIBADH, however, was active and displayed kinetic parameters similar to wild-type enzyme (Table 1), indicating that HIBADH may differ mechanistically from the other short-chain dehydrogenases studied to date. Mutation of this residue to alanine (Y162A) produced an enzyme which was inactive under all conditions tested. However, this enzyme was able to bind to the red agarose affinity resin and was specifically eluted with NAD^+. Therefore, this result was most likely due to a local structural effect, suggesting the importance of the bulk or size of tyrosine or phenylalanine at position 162. The exact role of Y162 in rat HIBADH is not known, but it is clear that this residue cannot be replaced by alanine which has a much simpler side chain. The effects of two other mutations, C163A and V166K, further indicated that this region may be structurally important for HIBA substrate binding. C163 is a semiconserved residue in that nearly all short-chain dehydrogenases contain a cysteine, serine, or threonine at this position which is immediately adjacent to the conserved tyrosine (Figure 2). C163A mutant HIBADH displayed an approximately 7-fold increase in K_b (K_m for HIBA) with no change in V_{max} or K_a (K_m for NAD^+), as shown in Table 1. This mutant also displayed a 5−6-fold increase in K_{ib} (496

Table 1: Kinetic Parameters of Wild-Type and Mutant HIBA Dehydrogenases[a]

enzyme	V_{max}, units/mg	K_m(HIBA), μM	K_m(NAD^+), μM
wild-type	10.3 ± 0.8	59 ± 6	24 ± 5
Y162F	10.0 ± 1.1	52 ± 5	23 ± 9
Y162A	–	–	–
Y32F	10.5 ± 1.3	55 ± 4	27 ± 3
V166K	–	–	–
C39A	7.8 ± 1.0	53 ± 4	25 ± 6
C155A	9.7 ± 0.9	56 ± 7	23 ± 5
C163A	10.5 ± 0.7	409 ± 23	29 ± 7
C175A	9.4 ± 0.9	57 ± 6	26 ± 4
C215A	–	–	–
C215D	–	–	–
C215S	1.8 ± 0.2	55 ± 8	28 ± 5
C278A	10.5 ± 1.2	58 ± 7	25 ± 4

[a] HIBADH activities and kinetic parameters were determined as described in Experimental Procedures. Values are mean ± SD for three determinations. Dashes indicate no detectable activity or that the parameter could not be measured. The lower limit for detection of activity was 0.1 ng of wild-type enzyme corresponding to a rate of 0.08 nmol/min.

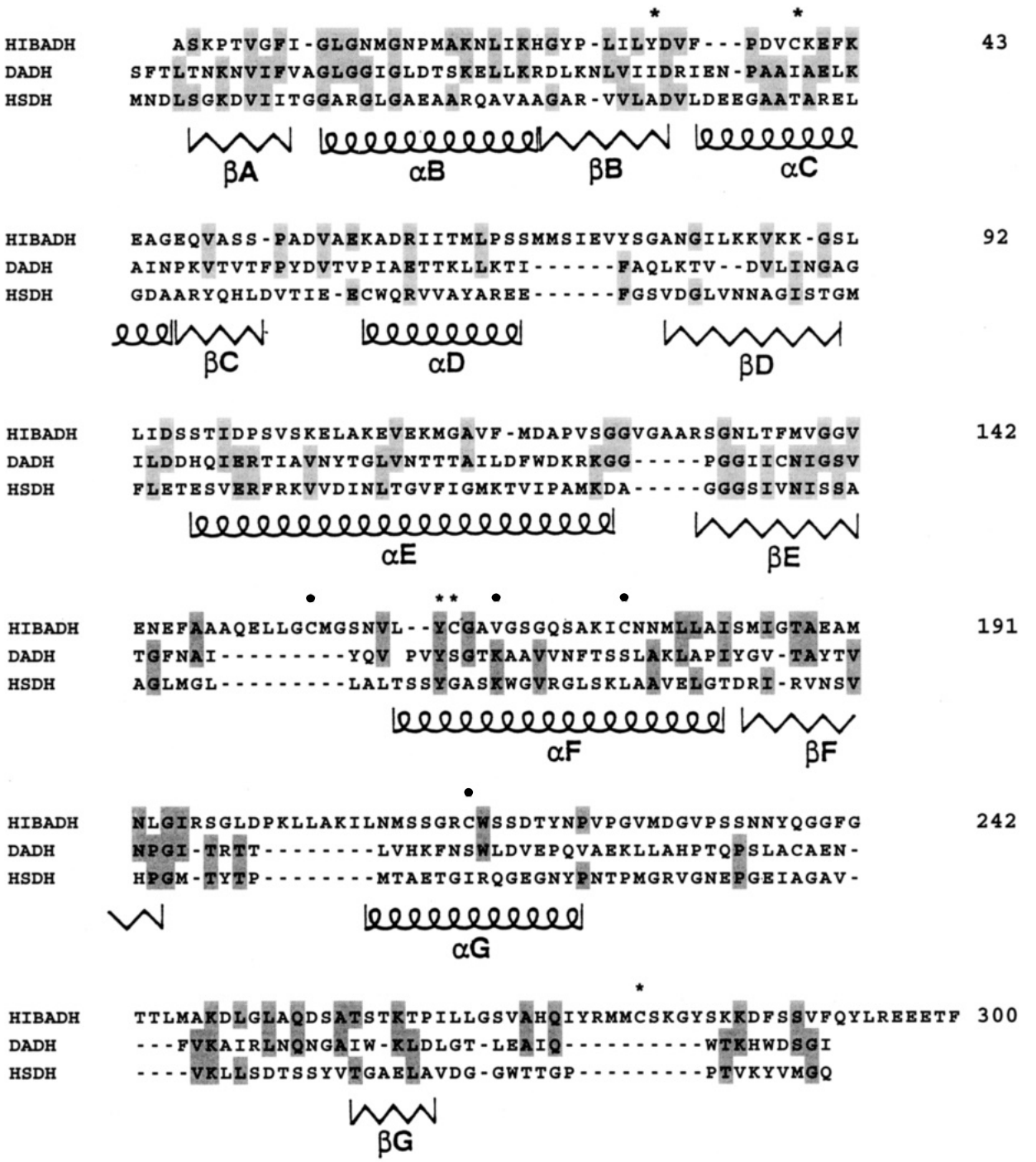

FIGURE 2: Alignment of amino acid sequences of HIBADH, *Drosophila* ADH, and 3α,20β-hydroxysteroid dehydrogenase. The amino acid sequences of HIBADH and *Drosophila* ADH (DADH) were aligned (Crabb et al., 1993) allowing further alignment of the 3α,20β-hydroxysteroid dehydrogenase (HSDH) amino acid sequence. Also shown is the previously reported secondary structure of HSDH (Ghosh et al., 1991). α-Helices are indicated by coils, and β-sheets are indicated by zigzags. Amino acid residues mutated in this study are indicated with stars.

and 90 μM for C163A and wild-type, respectively) but no change in K_{ia} (28 and 25 μM for C163A and wild-type, respectively). These data indicate that this residue was not crucial for catalysis to occur but may be involved in substrate binding, rather than in product turnover, or the release of products from the enzyme. Most other short-chain dehydrogenases contain a lysine exactly four residues C-terminal to the conserved tyrosine, whereas HIBADH contains a valine residue (V166) at this position (Figure 2). Site-directed mutagenesis has also been used to demonstrate a critical function for this conserved lysine residue in some short-chain dehydrogenases (Chen et al., 1993). When V166 of HIBADH was mutated to lysine, an inactive enzyme was produced. This mutant enzyme also bound to the red agarose affinity resin and was specifically eluted by NAD^+. Again, this suggests that this region of the enzyme is important to catalysis by HIBADH and that this residue may be critical for substrate binding or catalysis.

Tyrosine-Specific Chemical Modification. Reversible protection of cysteines by S-sulfonation and subsequent treatment with tetranitromethane has previously been used to demonstrate the importance of tyrosine residues for structure or catalytic function (Prozorovski et al., 1992). In this study we found that wild-type HIBADH could be reversibly

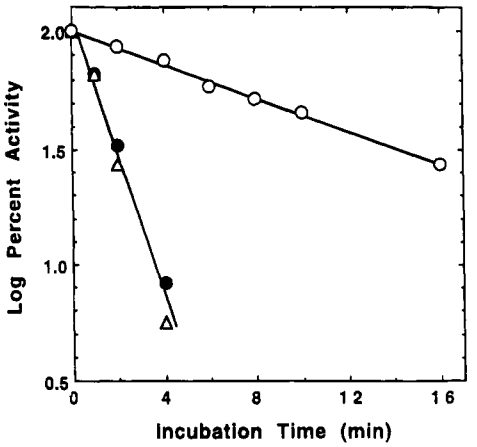

FIGURE 3: Inactivation of wild-type and mutant HIBADHs with *N*-acetylimidazole. Aliquots of wild-type (●), Y162F (△), and Y32F (○) HIBADHs were preincubated at room temperature with 1 mM *N*-acetylimidazole in buffer A. At the indicated time points, samples were removed and immediately assayed for HIBADH activity as described in Experimental Procedures. Values are averages of triplicate determinations.

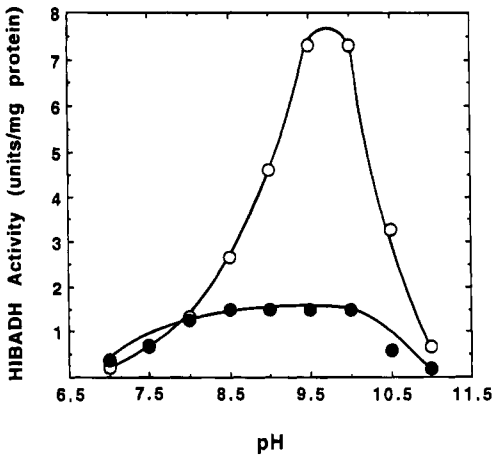

FIGURE 4: Effect of assay pH on wild-type and C215S mutant HIBADH activities. HIBADH activities of wild-type (○) and C215S (●) enzymes were determined exactly as described in Experimental Procedures with 100 mM Tris HCl (pH 7.0−8.0) and 100 mM NH_4Cl/NH_4OH (pH 8.5−11.0). Enzyme activities were unchanged when assays were performed with higher buffer or salt concentrations and with other buffers at the same pH values, indicating the absence of any buffer or salt effect. Values are averages of triplicate determinations.

inactivated by cysteine S-sulfonation with sodium sulfite and that regeneration of activity from sulfonated enzyme was completely prevented by prior treatment with tetranitromethane (data not shown). Further studies employed the use of *N*-acetylimidazole, a reagent reported to be more specific for tyrosine modification, since it produces *O*-acetyltyrosine without oxidizing cysteine sulfhydryl groups (Riordan et al., 1972). Treatment of the Y162F mutant HIBADH with *N*-acetylimidazole, in the presence of excess 2-mercaptoethanol, resulted in complete inactivation with a first-order rate constant (0.26 min^{-1}) identical to that for wild-type enzyme (Figure 3). However when tyrosine 32, which exists adjacent to a highly conserved aspartate in the putative NAD^+-binding domain, was mutated to phenylalanine, the mutant enzyme was inactivated by treatment with *N*-acetylimidazole but with a 7-fold slower rate (first-order rate constant of 0.04 min^{-1}) than that for the wild-type enzyme (Figure 3). The activity and kinetic parameters of Y32F HIBADH were similar to those of wild-type enzyme (Table 1), suggesting that this residue was not critical to catalytic function but that its modification may account, in part, for the inactivation of HIBADH by *N*-acetylimidazole, perhaps through structural interferences in the NAD^+-binding domain.

Site-Directed Mutants: Cysteine Residues. To assess the roles of each specific cysteine residue in catalysis by HIBADH, each of the remaining cysteines (C39, C155, C175, C215, and C278) was mutated to alanine. Four of these mutant enzymes were active and displayed kinetic parameters similar to wild-type enzyme (Table 1). However, C215A mutant HIBADH was completely inactive. To further examine the possibility of a catalytic function for this residue, mutations were made that gave amino acids with side-chain pK_a values greater and less than that of cysteine. When C215 was mutated to aspartate (pK_a = 3.86), the enzyme was inactive under all conditions tested, including a variety of pH values. This result could reflect either incompatible acid/base properties for the role of this residue in catalysis or that the size and stereochemistry of aspartate at position 215 are too greatly altered from that of cysteine. In contrast, C215S mutant HIBADH is catalytically active, with values for K_b and K_a (K_m for HIBA and NAD^+, respectively) similar to those of wild-type enzyme but with a 5-fold decrease in V_{max} (Table 1). Values for K_{ia} (29 μM) and K_{ib} (87 μM) were also similar to those of wild-type and thus unaffected by this mutation. From the known binding order of this enzyme-catalyzed reaction and the definitions of K_a, K_b, K_{ia}, and K_{ib}, these data would suggest that the decrease in the reaction velocity observed in the C215S mutant enzyme most likely results from a change in the reaction mechanism subsequent to the turnover of substrate to product, i.e., the kinetic constants for binding of methylmalonate semialdehyde or NADH. Furthermore the pH profile of C215S HIBADH activity was similar to that for wild-type enzyme only in the range of pH 9−11. In the range of pH 7.5−9.0, wild-type enzyme activity decreased steadily with decreasing pH, reaching a value nearly equal to that of the C215S mutant, whereas the mutant enzyme activity is practically unchanged in this pH range (Figure 4). The affect of pH on kinetic constants for each specific step of the sequential reaction mechanism catalyzed by HIBADH is not known.

Chemical Modification of Cysteine Mutants. Previous studies of rabbit liver HIBADH showed that this enzyme is highly sensitive to inactivation by various thiol-reactive compounds and that native enzyme could be protected from such inactivation by the presence of NAD^+ (Rougraff et al., 1988). Recombinant, wild-type HIBADH was completely inactivated when treated with 5 mM iodoacetate for 15 min at 25 °C, and equivalent treatments in the presence of 200 μM NAD^+ resulted in only 20% inactivation (inactivation defined as loss of total units of enzyme activity). Furthermore, each of the five active cysteine to alanine mutants (C39A, C155A, C163A, C175A, and C278A) was as sensitive to iodoacetate as the wild-type enzyme, both in the presence and absence of NAD^+. In contrast, C215S mutant HIBADH was completely insensitive to inactivation by iodoacetate but was inactivated by treatment with PMSF. As shown in Figure 5, C215S mutant HIBADH was inactivated by treatment with PMSF (first-order rate constant of 0.04 min^{-1}) with significant protection in the presence of

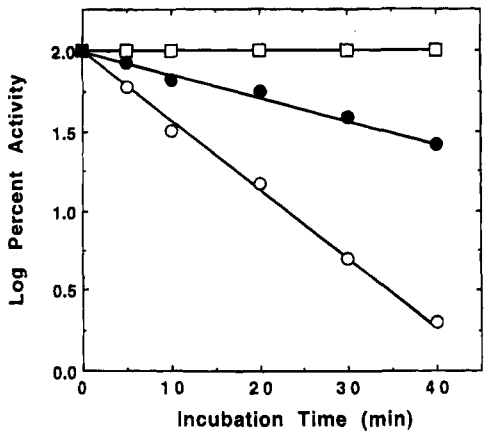

FIGURE 5: Inactivation of wild-type and C215S mutant HIBADHs with PMSF. Samples of wild-type HIBADH (□), C215S mutant HIBADH (○), and C215S mutant HIBADH plus 200 μM NAD^+ (●) were preincubated at room temperature in buffer A. After the addition of PMSF (3 mM final concentration from a concentrated stock solution in 2-propanol that gave a final concentration of 5% 2-propanol), aliquots were removed and immediately assayed for HIBADH activity. Control incubations with only 2-propanol added gave no change in activity. Values are averages of triplicate determinations.

NAD^+ (first-order rate constant of 0.01 min^{-1}), whereas wild-type enzyme was unaffected under these conditions. This suggested that inactivation by PMSF most likely occurred through reaction with the serine at position 215 and that this residue, though distal to the C-terminal NAD-binding domain, is most likely present at or near the NAD-binding site in the tertiary structure of the enzyme. Thus, mutation of C215 to serine converted HIBADH from a thio enzyme to a functional hydroxy enzyme, further supporting a catalytic role for this residue.

CD Spectrapolarimetry. To determine if mutations resulting in inactive enzymes caused only local chemical effects or gross structural changes (denaturation or improper folding), CD spectrapolarimetry was used to compare the apparent secondary structure contents of the purified recombinant wild-type and inactive mutant enzymes. Each of the enzymatically inactive mutants (Y162A, V166K, C215A, and C215D) produced spectra identical to that of the wild-type enzyme, indicating that these proteins did not grossly differ in secondary structure. Secondary structure contents were estimated to be 33% α-helix and 45% β-sheet for each of these enzymes, which agree well with that predicted by the sequence alignment of other enzymes with known structure (Figure 2).

DISCUSSION

HIBADH has a number of features common to the short-chain alcohol dehydrogenases. These enzymes do not require metal ions for catalysis, share sequence homology, and have a common domain structure consisting of an N-terminal NAD^+-binding domain and presumably a more centrally located substrate-binding domain. Since treatment of recombinant HIBADH with *N*-acetylimidazole or tetranitromethane produced time-dependent inactivation of the enzyme, we predicted that the conserved tyrosine residue Y162 may have a catalytic function, as was proposed for other short-chain alcohol dehydrogenases. However, the present data indicate that HIBADH acts through a different mechanism perhaps involving cysteine 215 as a general base catalyst. This hypothesis is supported by the activity and kinetic parameters of the Y162F mutant enzyme and the altered effects of chemical modification and pH on the C215S mutant enzyme. That this mutant can be completely inactivated by PMSF, whereas the wild-type enzyme is not inactivated by PMSF and is inactivated, instead, by sulfhydryl reagents, suggests that this residue may be important for catalysis or may be located at the active site. Furthermore, considering the acid/base properties of cysteine and serine, the lower rate of reaction and the relative insensitivity of the C215S mutant to changes in pH in the range of pH 8−10 are consistent with this residue functioning as a general base. Thiols are considerably weaker bases than alcohols; therefore, it would be expected that serine would be less easily deprotonated in the range of pH 8−10 as compared to cysteine whose pK_a value lies in this range. Cysteine may be more efficient as a base catalyst for proton abstraction in the oxidation of HIBA since it may be more easily protonated and deprotonated. Furthermore, the HIBADH-catalyzed reaction is reversible (Manning & Pollitt, 1985), and thus the base catalyst in the forward reaction must also act as an efficient acid catalyst in the reverse reaction. This task could be more efficiently performed by a cysteine residue as compared to a serine residue or a tyrosine residue. It is possible that this residue is part of a "proton relay system" or catalytic triad as exemplified by a number of other oxidoreductases (Eklund et al., 1982); however, the identities of other residues that might be involved in this system are not known. Further studies of these mutants and other residues possibly involved in the active site will be required to unambiguously determine the mechanism of catalysis by HIBADH.

Whether the short-chain alcohol dehydrogenase family can be subdivided on the basis of catalytic mechanism remains to be seen. However, it should be noted that one other member of this family, (*R*)-3-hydroxybutyrate dehydrogenase (BDH), is reported to be sensitive to cysteine modifications, and like HIBADH, it contains six cysteine residues. Furthermore, several of these cysteine residues can be aligned with cysteine residues in HIBADH, including C39, C163, and C175. Latruffe et al. (1980) reported that reaction of BDH with *N*-ethylmaleimide results in parallel loss of both enzyme activity and nucleotide cofactor binding. Selective modification with various other reagents led to the proposal that a single sulfhydryl group located near the C-terminal end of the BDH peptide is required for nucleotide cofactor binding but is not involved in catalysis (Dalton et al., 1993). However, no cysteine residue homologous to C215S of HIBADH is present in BDH.

Although the results of mutations of Y162 and C215 in HIBADH were somewhat unexpected, the present data are not necessarily in conflict with the current knowledge of the domain structure of short-chain alcohol dehydrogenases. A putative domain structure for HIBADH was obtained by comparing amino acid sequence alignments with the known secondary structure of 3α,20β-hydroxysteroid dehydrogenase (Figure 2). This alignment would suggest 36% α-helix in HIBADH, which agrees well with the α-helix content predicted from the CD spectra. HIBADH is 45 residues longer than either the 3α,20β-hydroxysteroid dehydrogenase or *Drosophila* ADH. It is interesting that the locations of the extra residues are found in the loops between α-helices and β-sheets. The locations of the mutated cysteines are

also of interest. Cysteine 39 is found within the putative nucleotide-binding domain, where it might not be expected to have a catalytic function. The remainder of the cysteines are clustered in the C-terminus of the enzyme that is expected to contribute most to the catalytic domain. Only cysteines 155 and 163 lie within regions that participate in formation of the substrate-binding pocket of the hydroxysteroid dehydrogenase. However, cysteine 155 is situated in a 9-residue insertion between β E and α F and hence may be displaced away from the active site. Cysteine 163 almost certainly contributes to the substrate-binding pocket, on the basis of the topology of the 3α,20β-hydroxysteroid dehydrogenase, and thus when C163 is replaced with alanine it is not surprising that an alteration in substrate binding or catalysis would be observed. In this case, the K_m for HIBA was markedly increased. In the sequence alignment, C215 is located in the α G helix. Thus, our data would suggest that this region contributes to the topology of the active site of HIBADH. It will be necessary to determine the crystal structure of this enzyme to understand the exact role of the cysteine 215 residue. In this regard, various conditions for crystallization of the recombinant enzyme have been found (unpublished data), but X-ray quality crystals have not been obtained.

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