Structural and mechanistic similarities of 6-phosphogluconate and 3-hydroxyisobutyrate dehydrogenases reveal a new enzyme family, the 3-hydroxyacid dehydrogenases

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Abstract Rat 3-hydroxyisobutyrate dehydrogenase exhibits significant amino acid sequence homology with 6-phosphogluconate dehydrogenase, D-phenylserine dehydrogenase from *Pseudomonas syringae*, and a number of hypothetical proteins encoded by genes of microbial origin. Key residues previously proposed to have roles in substrate binding and catalysis in sheep 6-phosphogluconate dehydrogenase are highly conserved in this entire family of enzymes. Site-directed mutagenesis, chemical modification, and substrate specificity studies were used to compare possible mechanistic similarities of 3-hydroxyisobutyrate dehydrogenase with 6-phosphogluconate dehydrogenase. The data suggest that 3-hydroxyisobutyrate and 6-phosphogluconate dehydrogenases may comprise, in part, a previously unrecognized family of 3-hydroxyacid dehydrogenases.

Key words: Dehydrogenase; 3-Hydroxyisobutyrate; 6-Phosphogluconate; Pyridoxal phosphate

1. Introduction

The catabolism of valine, unlike that of other branched-chain amino acids, occurs with the formation of a free branched-chain acid, (*S*)-3-hydroxyisobutyrate or HIBA, which is not metabolized as a coenzyme A thioester. HIBA is reversibly oxidized in mitochondria to methylmalonate semialdehyde by a highly specific, NAD^+-dependent dehydrogenase (HIBADH or 3-hydroxy-2-methylpropionate:NAD^+ oxidoreductase, EC 1.1.1.31) [1]. Native rabbit liver HIBADH and recombinant rat HIBADH have been purified and characterized [2,3], as well as several HIBADH isoforms from microbial sources [4,5], and cDNA sequences have been reported for the rat liver and *Pseudomonas aeruginosa* enzymes [6,7]. Previous studies of rat HIBADH tentatively placed the enzyme in the now well-established short-chain alcohol dehydrogenase family. This assignment was based on amino acid sequence homology, effects of tyrosine-specific chemical modification, and enzymatic properties such as the lack of a metal requirement for catalysis [3,8]. However, site-directed mutagenesis studies indicated that HIBADH differs in mechanism from the short-chain dehydrogenases studied to date, such as *Drosophila* alcohol dehydrogenase, and, therefore, may in fact not be a member of the short-chain dehydrogenases [3]. Indeed, the short-chain dehydrogenases mostly prefer secondary alcohols as optimal substrates whereas HIBADH is only active with primary alcohol substrates. We now show that HIBADH shares better amino acid sequence homology and enzymatic properties with a separate, previously unrecognized family of enzymes that includes 6-phosphogluconate dehydrogenase from numerous species, D-phenylserine dehydrogenase from *P. syringae*, and several hypothetical proteins of microbial origin. Like the short-chain dehydrogenases, these enzymes have no requirement for divalent metal ions, and contain a highly conserved dinucleotide cofactor-binding domain located at the N-terminus. However, the highly conserved sequence present in this domain differs from that of other dehydrogenases such as the short-chain dehydrogenases. The X-ray crystal structure of native sheep 6-phosphogluconate dehydrogenase was recently reported [9] and most of the amino acid residues suggested to be important for substrate binding and catalysis from the crystallographic data are completely conserved in all of the present sequences. Furthermore, specific, conserved lysine and asparagine residues proposed to be of importance in the catalytic mechanism of 6-phosphogluconate dehydrogenase [9] are now shown by site-directed mutagenesis to be important for catalysis by HIBADH. The present study suggests that 6-phosphogluconate dehydrogenase and HIBADH may share a common evolutionary origin and enzymatic mechanism.

2. Materials and methods

2.1. Materials

The oligonucleotide-directed mutagenesis system and *Escherichia coli* strain TG1 were obtained from Amersham. The Sequenase DNA sequencing system was obtained from US Biochemicals. The plasmid pET28a+ and the *E. coli* strain HMS174(DE3) were purchased from Novagen. VCS-M13 helper phage was obtained from Stratagene. Nickel-NTA-agarose was purchased from Qiagen. Methyl esters of (*S*)- and (*R*)-3-hydroxy-2-propionate were purchased from Aldrich. (*S*)- and (*R*)-3-hydroxyisobutyrate phenylethylamine salts were prepared as described previously [2]. All other chemicals were purchased from Sigma.

2.2. Construction of pET-HIBADH expression vector and site-directed mutagenesis

Rat HIBADH cDNA was excised from the previously described vector pGEX-HIBADH [3] by treatment with *Nde*I and *Hin*dIII and purified by agarose gel electrophoresis. This cDNA was ligated to the *Nde*I and *Hin*dIII sites of plasmid pET28a+ forming an expression vector coding for HIBADH with a 5′ 6-histidine tag fused in frame at the N-terminus. The fidelity of this construct was verified by dideoxynucleotide sequencing. Single stranded pET-HIBADH DNA was isolated using VCS-M13 helper phage as previously described [3]. Site-directed mutagenesis was performed according to the manufacturer's protocol (Amersham). The fidelity of each mutant cDNA was confirmed by restriction analysis and double-stranded sequencing. For expression of wild-type and mutant HIBADHs, the corresponding plasmids were transformed into HMS174(DE3) *E. coli* and grown in TY media at 37°C until the absorbance at 600 nm reached approx. 0.8. Isopropyl-β-D-thiogalactoside was added to a final concentration

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of 0.75 mM and cell growth was continued for 15–18 h. Recombinant enzymes were isolated from these cultures and purified according to previously published procedures [10]. The purity of each final preparation was assessed by SDS-polyacrylamide gel electrophoresis.

2.3. Enzyme assay and kinetic analysis

HIBADH activity was measured spectrophotometrically as previously described [3]. Production of NADH was measured by absorbance at 340 nm in a Cary 1E spectrophotometer. HIBA, NAD^+ and $NADP^+$ concentrations were measured as described previously [3]. Bisubstrate kinetic parameters were calculated using the computer program of Cleland [11].

2.4. Chemical modification

Purified HIBADH (0.5 mg) was treated with 5 mM pyridoxal phosphate in the presence or absence of 2.5 mM $NaBH_3CN$ at room temperature in a buffer consisting of 20 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 5 mM 2-mercaptoethanol. Reaction mixtures and stock reagents were protected from light with aluminum foil. At various times as indicated, samples were withdrawn and immediately used to determine HIBADH activity in previously mixed assay cuvettes. Control samples were incubated under identical conditions. To determine the reversibility of the inactivation, samples were dialyzed for 15 h at 4°C and in the dark in the above buffer.

2.5. 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 calculated using the reference spectra of Yang et al. [12] and the SSE-338 program (Japan Spectroscopic Co., Tokyo, Japan).

2.6. Other methods

Protein concentrations were determined using the Bradford protein assay [13] with bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [14].

3. Results and discussion

3.1. Amino acid sequence homology

The HIBADHs of both rat and *P. aeruginosa* display significant homology to 6-phosphogluconate dehydrogenase throughout their entire amino acid sequences. Fig. 1 shows an alignment of the HIBADH sequences and 5 hypothetical, bacterial proteins (unidentified ORFs) with the sequences of 6-phosphogluconate dehydrogenase from sheep, *S. cerevisiae*, and *E. coli*. Also shown is the N-terminal sequence of a specific D-phenylserine dehydrogenase purified from *P. syringae* [15]. Pairwise alignments indicate a range from 21%, for HIBADH vs. 6-phosphogluconate dehydrogenase, to over 70% identity with different combinations of these sequences. HIBADH shares somewhat higher homology with the bacterial

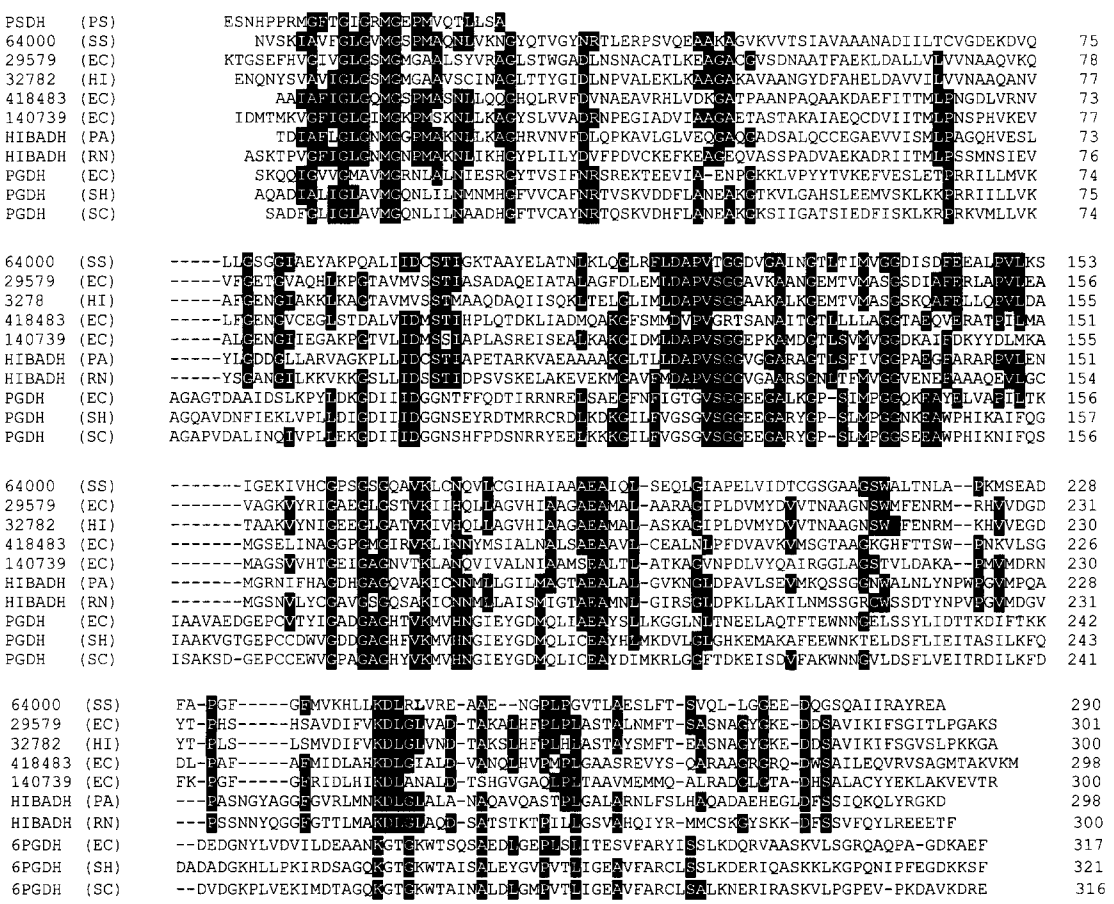

Fig. 1. Amino acid sequence comparison of HIBADH with D-phenylserine dehydrogenase, 6-phosphogluconate dehydrogenase, and five hypothetical bacterial proteins. All sequences were obtained from NCBI databases and alignments were performed using the NCBI BLAST network service by the method of Altschul et al. [18]. Residues conserved in 50% or more of the sequences are shown with reversed text. PSDH (PS) indicates D-phenylserine dehydrogenase from *Pseudomonas syringae*. HIBADH (PA) and HIBADH (RN) denote 3-hydroxyisobutyrate dehydrogenases from *Pseudomonas aeruginosa* and *Rattus norvegicus*, respectively. PGDH (EC), PGDH (SH), and PGDH (SC) indicate 6-phosphogluconate dehydrogenase from *Escherichia coli*, sheep, and *Saccharomyces cerevisiae*, respectively. Hypothetical proteins are identified by Genbank accession numbers. SS, EC, and HI indicate *Synechocystis* sp., *Escherichia coli*, and *Haemophilus influenzae*, respectively.

Table 1
Kinetic parameters of wild-type and mutant HIBADH[a]

Enzyme	V_{max} (units/mg)	K_a (μM)	k_{cat}/K_a (mM^{-1} s^{-1})	K_b (μM)	k_{cat}/K_b (mM^{-1} s^{-1})
NAD^+					
Wild-type	11.0 ± 0.8	22 ± 6	625 ± 110	56 ± 7	240 ± 30
D33R	0.7 ± 0.1	1240 ± 110	0.70 ± 0.08	112 ± 9	7.9 ± 0.7
N177Q	0.40 ± 0.03	55 ± 6	9 ± 1	890 ± 70	0.60 ± 0.05
$NADP^+$					
Wild-type	3.0 ± 0.4	960 ± 90	4 ± 1	104 ± 14	38 ± 5
D33R	2.0 ± 0.2	240 ± 60	10 ± 2	110 ± 12	22 ± 3
N177Q	–	–	–	–	–

[a]Values are mean ± S.D. for three determinations. Dashes indicate no measurable activity or that the parameter could not be determined. K_a and K_b represent the K_m values for dinucleotide cofactor and substrate, respectively.

hypothetical proteins than with 6-phosphogluconate dehydrogenase. However, the level of homology between the bacterial proteins alone suggests that these sequences most likely do not represent a single bacterial enzyme such as HIBADH. HIBADH, as well as each of the hypothetical, bacterial proteins are approx. 300 amino acid residues in length; whereas 6-phosphogluconate dehydrogenases from various species range in size from 468 to 482 residues. The D-phenylserine dehydrogenase was reported to be a dimer of identical, 31 kDa subunits, similar to the size and subunit composition of HIBADH [2,15]. The C-terminal third of the 6-phosphogluconate dehydrogenases (not shown in Fig. 1) comprises a domain absent from the other related enzymes and this domain exists in the reported crystal structure as a 'tail' which makes specific contacts with the phosphate moiety of 6-phosphogluconate, a part of the substrate absent in HIBA and phenylserine. Many of the most highly conserved residues are alanines and glycines which probably have important structural roles; however, several hydrophobic and polar residues, including two lysines and one glutamate, are strictly conserved. The N-terminus comprises one of the most highly conserved regions in all of these enzymes and shows a strict consensus sequence, **(A/G)XXGL(A/G)XMGX_5NX_4G**. This sequence is typical of the dinucleotide cofactor-binding fold

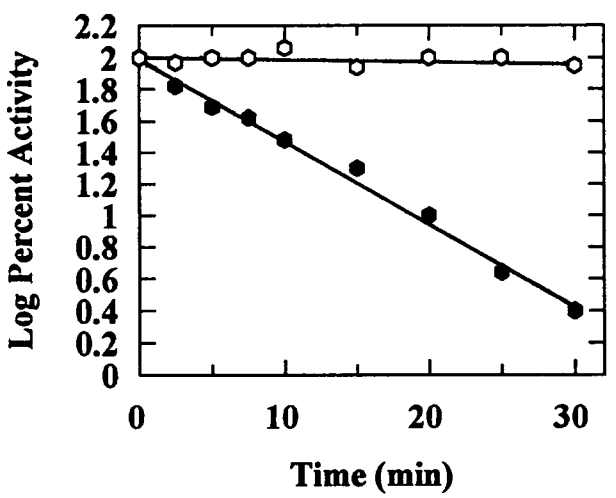

Fig. 2. Inactivation of HIBADH by pyridoxal phosphate. Recombinant HIBADH was treated with pyridoxal phosphate as described in Section 2. Closed symbols indicate enzyme treated with pyridoxal phosphate in the presence of sodium cyanoborohydride. Open symbols indicate enzyme treated with the reducing agent alone.

of many dehydrogenases, and this region of 6-phosphogluconate dehydrogenase is known to comprise the cofactor-binding domain from X-ray crystallographic data [9]. However, it differs slightly from the consensus sequenses found in other enzyme families such as the short-chain dehydrogenases [16]. Several other residues which have been implicated in substrate binding and catalysis in 6-phosphogluconate dehydrogenase also appear to be strictly conserved in this entire family of enzymes. These residues include Val-123, Ser-124, Gly-125, Gly-126, Lys-173, and Asn-177 (numbered according to rat HIBADH).

3.2. Site-directed mutagenesis

One of the most crucial residues in the cofactor-binding domain of dehydrogenases in general is a charged residue located approx. 18–20 residues C-terminal to the conserved GXGXXG motif which is preceded by several conserved hydrophobic residues [17]. An acidic residue such as aspartate at this position usually provides stringent specificity for NAD^+ whereas a basic residue such as arginine endows specificity for $NADP^+$. All of the published sequences for 6-phosphogluconate dehydrogenase, an $NADP^+$-specific enzyme, show an asparagine and an adjacent arginine at this position, and the crystal structure showed that this arginine is involved in binding $NADP^+$ [9]. HIBADH has an aspartate residue at this position, and is specific for NAD^+. To unambiguously localize this domain as the cofactor-binding domain of HIBADH, aspartate 33 was substituted with arginine. As shown in Table 1, this mutation produced a 56-fold increase in K_m (NAD^+) and a 900-fold decrease in k_{cat}/K_m (NAD^+), whereas K_m ($NADP^+$) actually decreased by 4-fold compared to wild-type. Therefore, the D33R mutation produced an enzyme with a grossly decreased catalytic efficiency with NAD^+, but with a reversal of the preference for NAD^+ vs. $NADP^+$. The crystallographic studies of sheep 6-phosphogluconate dehydrogenase also suggested that two important catalytic residues may be Lys-183 and Asn-187 (K173 and N177 in HIBADH). In the 6-phosphogluconate dehydrogenase-substrate cocrystal structure these two residues made hydrogen bonds to the 3-hydroxyl group of the substrate [9]. A mechanism was proposed whereby the asparagine residue acts as the donor in the hydrogen bond to the 3-hydroxyl, and the 3-hydroxyl acts as the donor in the hydrogen bond to the lysine residue. This lysine residue was proposed to act as a base catalyst. The level of homology seen with these residues in the presently reported enzymes suggested the possibility of a common mechanism. To test this mechanistic proposal, Lys-173 was substituted

Table 2
3-Hydroxyacid substrate specificity of HIBADH[a]

Substrate	K_m	V_{max} (units/mg)	k_{cat}/K_m (mM^{-1} s^{-1})
S-HIBA	59 ± 6 μM	10.3 ± 0.9	215 ± 22
R-HIBA	1.5 ± 0.1 mM	0.9 ± 0.1	0.89 ± 0.06
L-Glycerate	9.8 ± 0.8 mM	8.0 ± 1.0	1.06 ± 0.09
L-Serine	21 ± 1 mM	10.5 ± 1.2	0.64 ± 0.10
S-3-Hydroxybutyrate	–	–	–
L-Mevalonate	–	–	–

[a]Values are mean ± S.D. for three determinations. Dashes indicate no measurable activity or that the parameter could not be determined.

with alanine, arginine, asparagine, and histidine, and Asn-177 was substituted with glutamine. Each of the Lys-173 mutants was well expressed and purified without apparent proteolysis. However, each of these mutations produced inactive enzymes, even when assayed at very high protein concentrations, high concentrations of substrate, and various conditions of pH. Each of the Lys-173 mutant enzymes displayed circular dichroism spectra identical to that of wild-type enzyme, suggesting that no large changes in secondary structure or assembly of the enzymes resulted from these mutations. Thus, Lys-173 appears to play a crucial role in catalysis by HIBADH. Substitution of Asn-177 with glutamine produced an enzyme which retained catalytic activity, but displayed a 27-fold decrease in V_{max}, and a 16-fold decrease in K_m (HIBA) (Table 1), but no change in K_m (NAD^+), consistent with the previously proposed catalytic role for this conserved residue involving a specific interaction with the 3-hydroxyl group of the substrate.

3.3. Lysine-specific chemical modification

Previous studies utilizing chemical modification of HIBADH showed the enzyme to be sensitive to chemical modifiers of cysteine and tyrosine [3]. We now report that recombinant rat HIBADH is also readily inactivated by treatment with pyridoxal phosphate. In the presence of 5 mM pyridoxal phosphate and 2.5 mM $NaBH_3CN$, at 25°C, the inactivation occurs with a first order rate constant of 0.05 min^{-1} (Fig. 2), and is irreversible upon extended dialysis, suggesting the formation of a stable Schiff base with a lysine residue. Treatment with pyridoxal phosphate in the presence and absence of cofactor and substrates showed significant protection against inactivation in the presence of saturating concentrations of either *R*- or *S*-HIBA, both of which are active substrates, but virtually no protection in the presence of NAD^+. In light of this finding it is interesting to note that the crystal structures of 6-phosphogluconate dehydrogenase in the presence and absence of bound cofactor indicated a rigid structure with no change in conformation upon cofactor binding [9]. The present data are consistent with the presence of a lysine residue, such as Lys-173, in the active site of rat HIBADH which may be involved in substrate binding although other lysine residues may be modified and could possibly contribute to inactivation of the enzyme.

3.4. Substrate specificity

3-Hydroxyisobutyrate, 6-phosphogluconate, and phenylserine have in common only the 3-hydroxyacid functionality, suggesting that this may be a common feature of the substrate specificity of the presently proposed family of enzymes. Previously, rabbit and rat HIBADH were reported to be specific for *S*-HIBA and *R*-HIBA though with approx. 20-fold increased K_m for the *R* enantiomer. Several microbial isozymes of HIBADH were reported to be also specific for HIBA but with differences in the enantiomeric specificity [5]. Further analysis of the substrate specificity of rat HIBADH indicated that the enzyme is active with a series of 3-hydroxyacid substrates including *S*- and *R*-HIBA, L-glycerate, and L-serine. The V_{max} with most of these substrates was approximately the same, however, the K_m increased with increasing polarity of the substituent on the 2-carbon (Table 2). The enzyme was inactive with similar compounds containing carbon backbones of four or five carbons in length (Table 2). Therefore, the optimal substrate can be defined as an L or *S*-3-hydroxyacid with a three-carbon backbone and a nonpolar substituent such as a methyl group on the 2-carbon. Substrate specificity studies of the bacterial D-phenylserine dehydrogenase also indicated that only 3-hydroxyacids (in this case, D-3-hydroxyamino acids) could serve as active substrates for this enzyme [15]. It is interesting to note that the majority of the sequences reported in the present study are of microbial origin, and may represent enzymes with particular importance in bacterial biochemistry. One of the bacterial ORFs (32782 from *Haemophilus influenzae*) was reported to be present on an operon induced by glyoxylate, and one of the enzymes common to the glyoxylate cycle of plants and bacteria, tartronate semialdehyde reductase, catalyzes a reaction very similar to that of HIBADH:

$$CH_2OHCH_2OHCOO^- + NAD^+ \leftrightharpoons CHOCH_2OHCOO^- + NADH + H^+$$

However, identification of this sequence as that of tartronate semialdehyde reductase is merely speculative at this time. Although several of these sequences remain as unidentified open reading frames, we propose that these homologous proteins represent a distinct family of enzymes most likely consisting of 3-hydroxyacid dehydrogenases.

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