NOVEL DEHYDROGENASE CATALYZES OXIDATIVE HYDROLYSIS OF CARBON-NITROGEN DOUBLE BONDS FOR HYDRAZONE DEGRADATION *

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Running Title: Novel mechanism for C=N oxidation

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Hydrazines and their derivatives are versatile artificial and natural compounds that are metabolized by elusive biological systems. Here we identified microorganisms that assimilate hydrazones and isolated the yeast, Candida palmioleophila MK883. When cultured with adipic acid bis-(ethylidene hydrazide) (AEH) as the sole source of carbon, C. palmioleophila MK883 degraded hydrazones and accumulated adipic acid dihydrazide. Cytosolic NAD\(^+\) - or NADP\(^+\)-dependent hydrazone dehydrogenase (Hdh) activity was detectable under these conditions. The production of Hdh was inducible by AEH and the hydrazone, varelic acid ethylidene hydrazide under the control of carbon catabolite repression. Purified Hdh oxidized and hydrated the C=N double bond of acetaldehyde hydrazones by reducing NAD\(^+\) or NADP\(^+\) to produce relevant hydrazides and acetate, the latter of which the yeast assimilated. The deduced amino acid sequence revealed that Hdh belongs to the aldehyde dehydrogenase (Aldh) superfamily. Kinetic and mutagenesis studies showed that Hdh formed a ternary complex with the substrates and that conserved Cys is essential for the activity. The mechanism of Hdh is similar to that of Aldh, except that it catalyzed oxidative hydrolysis of hydrazones that requires adding a water molecule to the reaction catalyzed by conventional Aldh. Surprisingly, both Hdh and Aldh from baker's yeast (Ald4p) catalyzed the Hdh reaction as well as aldehyde oxidation. Our findings are unique in that we discovered a biological mechanism for hydrazone utilization and a novel function of proteins in the Aldh family that act on C=N compounds.

Hydrazides are derivatives of acids in which a hydroxyl residue is substituted with a hydrazino (-NR1-NR2R3) group. Acyl derivatives of hydrazide, such as isonicotinic acid hydrazide and its related compounds are anti-tuberculosis reagents and some of them are also antidepressants (1). Among the naturally occurring hydrazides, argaritine, a phenyl derivative of hydrazide found in the commercial mushroom Agaricus bisporus might have carciogenic activity (2). Hydrazide derivatives that lack substitutions on one of the nitrogen atoms (R1R2N-NH2) possess a highly active -N-NH2 moiety and easily react with the carbonyl groups of various compounds to produce hydrazones, which can be described as:

\[
R_1R_2N-NH_2 + C(=O)R_3R_4 \rightarrow R_1R_2N=N=CR_3R_4 + H_2O
\]

This hydrazone-forming reaction is essentially a condensation reaction of the hydrazides and the carbonyl compounds. This reaction is applied for synthesizing versatile compounds including pharmaceuticals, dyes, agrochemicals and other organic compounds (3). Hydrazones are also distributed in nature. One example is the hemolytic toxin, gyromitrin (acetaldehyde methylformylhydrazone), which is produced by false morel mushrooms (4). An alkaloid-containing hydrazone has been isolated from a
marine sponge (5). However, their physiological functions and biosynthetic mechanisms are unknown.

Despite their chemical and biological importance, few biological systems metabolize hydrazides and hydrazones. The liver might metabolize isonicotinic acid hydrazide to isonicotinic acid through cytochrome P450 (6) and amidase (7) activities, but the enzymes that catalyze this reaction remain obscure. Earlier reports have shown that an actinomycete bacterium produces hydrazidase that hydrolyzes isonicotinic acid hydrazide and its derivatives (8). Agaritine γ-glutamyltransferase (EC 2.3.2.9) has been partially purified and is proposed as the enzyme responsible for the esterification of γ-glutamyl carboxylate to p-hydroxymethylphenylhydrazine to form agaritine (9). Although these enzymes attack or form acyl hydrazides, little is known about those that attack the double bond of the N-N=C hydrazone groups. To our knowledge, peptidylglycine hydroxylase (EC1.14.17.3) (10) and glutamine transaminase (EC2.6.1.15) (11) are the only known enzymes that react with hydrazones. However, hydrazones are not their physiological substrates, and thus, how biological systems utilize hydrazones remains obscure.

Here we chemically synthesized hydrazone compounds (adipic acid bis-(ethylidene hydrazide) (AEH)1, and varelic acid ethylidene hydrazide (VEH)) (Fig. 1A), isolated the yeast that assimilates them from soil, and purified an enzyme that degrades hydrazones. The enzyme attacked the C=N double bond of hydrazones and catalyzed NAD+-dependent oxidation and hydration to produce the relevant hydrazide and acid. This ‘oxidative hydrolysis’ reaction is unique among known enzymes. We also found that the enzyme belongs to the aldehyde dehydrogenase (Aldh) superfamily. The present study is the first to uncover a mechanism for hydrazone assimilation in biological systems and a novel role of an enzyme in the ALDH superfamily, namely cleavage of the C=N double bond.

**EXPERIMENTAL PROCEDURES**

**Reagents**— The hydrazones in Fig. 1 were prepared and identified as described in the Supplemental information. Purity (96% <) was confirmed by NMR. All other reagents were of biochemical analysis grade. *S. cerevisiae Ald4p* was obtained from Sigma (Cat No. A9770).

**Strains, Culture, and Media**— We isolated strain MK883 from soil after screening in carbon-limited minimal (MM) medium (10 mM NH₄Cl, 10 mM potassium phosphate (pH 7.2), 0.05% MgSO₄, 0.05% KCl, 0.2% Hunter’s trace element solution (12)) containing 30 mM AEH (MMAEH medium), and Luria Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl). We analyzed the effects of the carbon source by replacing 30 mM AEH with appropriate carbon sources. *C. palmioleophila* NBRC70761 and other type strains were obtained from the Biological Resource Center, National Institute of Technology and Evaluation, Japan unless otherwise stated and maintained in YMPD medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose).

**Screening of Hydrazone-degrading Microorganisms**— Soil samples were suspended in 5 ml of MMAEH medium in test tubes and shaken (120 rpm) at 30°C for 48 h. Fresh MMAEH medium was inoculated with 0.1 ml of the culture and incubated under the same conditions. These steps were repeated twice. The resulting suspension was streaked on LB plates and incubated at 30°C. Bacterial and yeast colonies that appeared on the plates were transferred to 5 ml of MM medium containing 30 mM sodium succinate or LB medium, incubated at 30°C for 24 h, and 1 ml was inoculated into 100 ml of MMAEH medium. After shaking (120 rpm) at 30°C for 20 h, cells were collected by centrifugation at 8,000 rpm, suspended in buffer A (50 mM potassium phosphate (pH 7.2), 10% glycerol, 0.1 mM dithiothreitol (DTT), 0.1 mM EDTA), and disrupted by ultrasonication (Sonifier 250,
Branson, CT). Homogenates were centrifuged at 15,000 rpm to remove debris and used for enzyme assays. Strains with high levels of enzyme activities were selected.

**Enzyme Assays**— Hydrazine dehydrogenase activity was measured using the following assay systems. *Method A*: The reaction mixture comprised 50 mM potassium phosphate buffer (pH 7.2), 5 mM AEH or VEH, 5 mM NAD\(^+\), and 1 mM DTT (100 µl total volume) and the reaction was started by adding enzyme. After incubation at 30°C for 15 to 120 min, the reaction was stopped by adding 100 µl of cold acetonitrile, and the mixture was centrifuged at 15,000 rpm for 5 min. The AEH remaining in the supernatant was measured using HPLC as described below. *Method B*: The reaction mixture used in *Method A* was placed in an optical cell and the reaction was started by adding enzyme. Increases in NADH were followed by monitoring absorbance at 340 nm at 25°C using a DU-7500 spectrophotometer (Beckman-Coulter Inc., CA). The molecular coefficient of 6.22 mM\(^{-1}\) cm\(^{-1}\) was used for NADH. Hydrolyzing activity of p-nitrophenyl acetate was measured in 50 mM potassium phosphate buffer (pH 7.2), 3 mM pNP acetate, 1 mM DTT and an appropriate amount of enzyme. Reactions proceeded at 30°C for 20 min and then the mixtures were centrifuged at 15,000 rpm for 5 min, and p-nitrophenol was measured as absorbance at 405 nm. The molecular coefficient of 1.71 × 10\(^4\) M\(^{-1}\) cm\(^{-1}\) was used for p-nitrophenol. Aldh activity was measured by *Method B* except that the reaction mixture comprised 50 mM sodium pyrophosphate/potassium dihydrogen phosphate buffer (pH 9.0), 0.025 mM acetaldehyde, 1 mM NAD\(^+\), 1 mM DTT. The activities of glucose 6-phosphate dehydrogenase and cytochrome c oxidase were assayed as described (13, 14). The protein concentration was determined using Protein Assay Kits (Bio-Rad Laboratories Inc., CA) with bovine serum albumin as the standard.

**Analytical Methods**— Levels of AEH, AMH, and ADH were determined using HPLC (HP-1100, Hewlett-Packard Co., CA) equipped with a TSKgel ODS-80TM column (4.6 x 150 mm; Tosoh, Tokyo, Japan), and by monitoring absorption at 210 nm. The mobile-phase solvent system comprised 50 mM potassium phosphate buffer (pH 7.2): acetonitrile (9:1 v/v) at a flow rate of 1.0 ml min\(^{-1}\). We separated VEH and VH using 50 mM potassium phosphate buffer (pH 7.2): acetonitrile (8:2 v/v) as a solvent. Acetate was determined by suppressor anion chromatography (Model 761, Compact IC, Metrohm, Switzerland) according to the manufacturer’s instructions.

**Purification of Hydrazine Dehydrogenase**— *C. palmioleophila* MK883 was incubated in 200 ml of LB medium at 30°C for 15 h. Cells were collected after centrifugation at 8,000 rpm for 15 min, transferred to MMAEH medium, incubated at 30°C for 20 h, and collected by centrifugation as above. Typically, 20 g of cells (wet weight) obtained from 20 liters of culture were suspended in buffer A, and homogenized with aluminum oxide. The homogenates were centrifuged at 15,000 rpm for 30 min, and the resulting supernatant was applied to a column containing DEAE cellulose (DE52, Whatman International Ltd., UK) equilibrated with buffer A. Proteins were eluted from the column with 300 ml of buffer A at a flow rate of 0.8 ml min\(^{-1}\). Fractions with hydrazine dehydrogenase activity were pooled, and applied to a column containing hydroxyapatite (Wako Pure Chemical Ind. Ltd., Japan) equilibrated with buffer A and then eluted with a linear gradient of potassium phosphate (0 to 0.5 M) in buffer A at a flow rate of 0.5 ml min\(^{-1}\). Each of these steps proceeded at 4°C. Fractions containing hydrazine dehydrogenase activity were pooled, dialyzed against buffer B (20 mM Tris-HCl (pH 8.0), 10% glycerol, 0.1 mM EDTA) for 16 h and then applied to Resource Q columns (Amersham Biotech) equilibrated with buffer B. Proteins were eluted with a linear gradient of KCl (0 to 0.5 M) in buffer B at a flow rate of 0.5 ml min\(^{-1}\). Fractions containing hydrazine dehydrogenase were applied to a Superose 6 10/300 GL column (Amersham Biotech) that was equilibrated with...
50 mM potassium phosphate buffer (pH 7.2), 150 mM NaCl, and eluted at a flow rate of 0.5 ml min⁻¹. Fractions containing hydrazone dehydrogenase activity were pooled and used as the purified preparation.

Characterization of hydrazone dehydrogenase—The molecular mass of the purified enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels as described by Laemmli (15) or by gel filtration chromatography using Superose 6 10/300 GL (Amersham Biotech) as above. The effects of inhibitors and metal ions (1 mM each) on the enzyme activity were examined after the enzyme (1 μg) was incubated with each reagent in 50 mM potassium phosphate (pH 7.2) for 1 h at room temperature. Steady-state turnover of hydrazone dehydrogenase activity was measured using Method B. Purified protein (1 μg) was resolved by SDS-PAGE and then proteins were electronically blotted onto a polyvinylpyrrolidone membrane. Blots were stained with Coomassie Brilliant Blue R-250, protein bands were excised, and the amino terminal amino acids were determined using an automated protein sequencer (Model Precise 492, Perkin Elmer).

Cloning and Nucleotide Sequencing—Total DNA of the strain MK883 served as the template for polymerase chain reaction (PCR) with the degenerate oligonucleotide primers FA, FB, FC, and FD (Table S1). The first PCR included 200 pM of primers FA and FB and 0.5 μg total DNA as the template with denaturation at 94°C for 10 min, followed by 30 cycles of 94°C for 0.5 min, 42°C for 1 min, 72°C for 2 min and a final extension at 72°C for 10 min. The second PCR proceeded using the product of the first PCR as the template and FC and FD as the primers under the same conditions.

Complementary DNA was synthesized using 5'-Rapid Amplification of cDNA Ends (RACE) version 2.0, and 3'-RACE systems (Invitrogen Co., CA) according to the manufacturer’s instructions. Total RNA was prepared from the strain MK883 cultured in MMAEH medium for 24 h as above. The gene specific primers for 5'- RACE were GSP1 and GSP2 and those for 3'-RACE were GSP3 and GSP4 (Table S1). The products were cloned into pGEM®-T easy (Promega, Madison, WI). The D1/D2 region of the gene encoding 26S rRNA (16), 1.7 kbp of 18S rRNA (17), and 1.4 kbp of 16S rRNA (18) were isolated as described previously. Nucleotide sequences were determined by using an automated DNA sequencer (CEQ2000, Beckman Coulter, Inc., CA) according to the manufacturer’s instructions. Nucleotide sequences will appear in the GenBank/EMBL/DDJB nucleotide database under accession numbers AB361432 (Hdh), AB361594 (26S rRNA of MK883), AB361592, AB361593 (18S rRNA of C. palmioleophila and Williopsis saturnus), AB361588, AB361589, AB361590, and AB361591 (16S rRNA of Pseudomonas putida, Delftia acidovorans, Bacillus flexus, P. aeruginosa).

Steady-state Kinetics—Kinetic constants for hydrazone dehydrogenase activity were assessed using data obtained with variable concentrations of one compound at a fixed concentration (1 mM) of another. Apparent $K_m$ values for AEH and NAD(P)$^+$ were determined by fitting each dataset to Equation 1.

$$v/e = k_{cat} [A] / (K_A + [A])$$ (Eq. 1)

Dehydrogenase activity for VEH was assayed with varied concentrations of VEH at fixed concentrations of NAD(P)$^+$. Assuming that the reaction mechanism was sequential (Fig. 5), the data were analyzed by non-linear regression (Origin ver 6, OriginLab) after fitting the data to Equation 2 and determining $K_{iB}$ from $K_{iB} = (K_{iA} K_B) / K_A$.

$$v/e = k_{cat} [A] [B] / (K_A K_B + K_A [B]$$

+ $K_B [A] + [A][B])$ (Eq. 2)

Data obtained from pNP acetate hydrolase inhibition by VEH and NAD$^+$ were fitted to Equation 3 for competitive inhibition or
Equation 4 for mixed inhibition, respectively.
\[
v/e = \frac{k_{\text{cat}} [A]}{(K_A (1 + [I] / K_i)+ [A])}
\]
(Eq. 3)

\[
v/e = \frac{k_{\text{cat}} [A]}{(K_A (1 + [I] / K_i)+ [A] [I] K_A / K_i K_{A'})}
\]
(Eq. 4)

In Equations 1 to 4, \(v\) = initial velocity, and \(e\), \([A]\), \([B]\), and \([I]\) are the concentrations of enzyme, substrates A and B, and inhibitor. \(K_A\) and \(K_B\) are the Michaelis-Menten constants of substrates A and B. \(K_A\) and \(K_B\) are the dissociation constant for substrates A and B. \(K_i\) is the inhibition constant. \(K_{A'}\) is the dissociation constant for the enzyme-substrate-inhibitor complex.

**Preparation of Recombinant Hdh**—For producing wild type Hdh with a 6× His tag on the amino terminus, the cDNA of Hdh was amplified by PCR using the primers HF and HR (Table S1), digested with NdeI and XhoI, and cloned into pET15b (Novagen, Germany). The plasmid was then digested using the same enzymes. The resultant plasmid (pET15hdh1) was introduced into *Escherichia coli* Rosetta gami B (DE3). Plasmids for producing the mutant Hdh were constructed by PCR-based site-directed mutagenesis (19) using the primers CSF1 and CSR1 for C301S mutant, and EAF1 and EAR1 for E267A mutant (Table S1). The *E. coli* strain was cultured at 120 rpm and 37°C for 12 h in LB medium containing 50 μg l⁻¹ sodium ampicillin (LA medium), and then 10 ml was transferred to 100 ml of LA medium. After incubating for 2 h at 37°C, 0.1 mM isopropyl thiogalactoside was added to the culture. Incubation was continued for additional 8 h at 50 rpm and 25°C. The cells were collected by centrifugation at 6,000 rpm for 10 min, suspended in buffer A, ultrasonicated, and centrifuged at 15,000 rpm for 30 min to remove cell debris. The resultant cell-free extract was applied to a chelating Sepharose column (φ 1 x 2 cm) and equilibrated with buffer C (buffer A containing 300 mM NaCl). Bound proteins were eluted with buffer C containing 500 mM imidazole. Resultant fractions were dialyzed against buffer A, and analyzed.

**Quantitative PCR**—Total RNA of the strain MK883 was extracted from cells cultured in MMAEH, and MM media containing 200 mM glucose, 30 mM sodium succinate, and both 30 mM AEH and 200 mM glucose as the sole carbon source at 30°C for 20 h. First-strand cDNA was synthesized by incubating total RNA (2.3 μg), in 10 μl of reaction buffer comprising Oligo (dT) 20 (Toyobo Co., Ltd, Japan), 5× reverse transcriptase buffer and reverse transcriptase M-MLV (200 units) (Takara Bio, Inc., Japan)) at 42°C for 90 min. First-strand cDNA (1 μl) synthesized in this reaction was amplified by quantitative PCR using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc., CA) and MiniOpticon™ version 3.1 (Bio-Rad Laboratories Inc., CA) according to the manufacturer’s instructions. The expression of Hdh was normalized against that of 18S rRNA. Data were calculated as relative expression (20). The primers were HRF and HRR for Hdh, and 18RF and 18RR for 18S rRNA (Table S1).

**Preparation of Subcellular Fractions**— *C. palmioleophila* MK883 cells were cultured in 300 ml of MMAE Rh medium at 30°C for 20 h, and collected by centrifugation as described above. Cells were suspended in buffer (1.2 M sorbitol, 20 mM KH₂PO₄/K₂HPO₄, pH 7.4) containing Zymolyase 20-T (Seikagaku Co., Japan), and incubated at 30°C for 30 min, centrifuged at 3,000 × g for 5 min, and washed with the same buffer. The resulting protoplasts were lysed with buffer (0.25 M sorbitol, 20 mM KH₂PO₄/K₂HPO₄, pH 7.4). The supernatant recovered after removal of cell debris (at 3,000 × g for 5 min) was further centrifuged at 10,000 × g for 5 min. The supernatant and precipitates suspended in the same buffer were used as cytosolic and mitochondrial fraction.

**RESULTS**

*Isolation and Identification of Novel*
**Hydrazone-assimilating Microorganisms**—Before screening microorganisms that assimilate hydrazone, we chemically synthesized the hydrazone compound, adipic acid bis-(ethylidene hydrazide) (AEH) (Fig. 1A, (3)), as described in the SUPPLEMENTAL INFORMATION since insufficient amounts of hydrazone are commercially available for our screening. Coupling the two hydrazide groups of adipic acid dihydrazide (ADH) (1) and acetaldehyde generated AEH with two hydrazone groups. We also prepared adipic acid ethylidene hydrazide (AMH) (2), which is derived from ADH when one hydrazide group remains unreacted with acetaldehyde. By enriching cultures in medium containing 30 mM AEH as the sole source of carbon (MMAEH medium), we screened 128 soil samples and isolated 82 microorganisms that grow by utilizing AEH as a carbon source. Seven strains expressed high levels of cell-free AEH-degrading activity (0.035 to 1.86 nmol min⁻¹ mg⁻¹). The reaction produced AMH and ADH, indicating that the isolates reacted against the C=N double bonds of both the hydrazone moieties of AEH. We selected one of the strains, MK883, that rapidly proliferated and produced the most AEH-degrading activity for further analysis.

Strain MK883 was cultured in MMAEH medium (Fig. 2A). At the initial stage of the culture (~ 15 h), AEH concomitantly decreased with increasing AMH and ADH. Further culture (15 ~ 23 h) decreased the accumulated AMH and increased ADH, indicating that MK883 metabolized AEH to AMH, and AMH to ADH. The reaction was stoichiometric regarding the amounts of AEH, AMH, and ADH, which also supported the reaction scheme 4 in Fig. 1B. The optical density of the culture was increased as AEH was consumed, indicating that AEH degradation conferred an increase in cell mass. Strain MK883 degraded the hydrazone, varelic acid ethylidene hydrazide (VEH; see Fig. 1A, (5) and SUPPLEMENTAL INFORMATION) to produce varelic acid hydrazide (VH) (Fig. 2B). Since no carbon source other than AEH or VEH was included in the culture medium, these results indicated that strain MK883 metabolized the hydrazones (AEH and VEH) as both carbon and energy sources.

The nucleotide sequence for the D1/D2 region of the 26S rRNA-coding gene of strain MK883 precisely matched that of *C. palmioleophila* NRRL Y-17323 (accession number U45758) and *C. palmioleophila* (strain name unspecified) (DQ234655). Strain MK883 had the morphology of a budding yeast and formed pseudohyphae during vegetative growth. No sexual reproductive organs were formed within 30 days of incubation. Strain MK883 did not ferment glucose, and grew aerobically with any of glucose, galactose, sucrose, maltose, trehalose, melibiose, raffinose, melezitose, sorbitol, D-mannitol, 2-keto-D-gluconate, DL-lactate, citrate, and ethanol as the sole carbon source. However, the strain did not grow in the presence of L-sorbose, D-ribose, L-arabinose, cellobiose, salicin, lactose, erythritol, galactinol or inositol. Strain MK883 did not grow with nitrate as a sole nitrogen source but thrived in the presence of nitrite, L-lysine or cadaverine. These properties are consistent with those of *Candida palmioleophila* (21), so we designated the isolate *C. palmioleophila* MK883.

**Purification of Enzyme that Degrades Hydrazone**—Cell-free extracts of *C. palmioleophila* MK883 had AEH-degrading activity that was completely lost after dialyzing the cell-free extract against buffer A. Adding either NAD⁺ or NADP⁺ (1 mM each) to the dialysate recovered the activity although other cofactors (FAD, FMN, α-lipoic acid, CoA, acetyl-CoA, biotin, 1 mM each) were inactive (data not shown). We also found that absorption at 340 nm increased as the reaction proceeded. This showed that NAD⁺ was reduced to NADH during the reaction, and thus we measured enzyme activity by following the increase in the absorbance (Method B in EXPERIMENTAL PROCEDURES) for purification. We purified the AEH-degrading activity 230-fold with 8.7% recovery from cell-free extracts of *C. palmioleophila* MK883 using four chromatographic separations (Table 1), each of
which resulted in a single peak of activity. Resolution as a single band (59 kDa) on SDS-PAGE confirmed the homogeneity of the purified preparation (Fig. 3). The molecular mass \( M_r \) of the enzyme calculated from gel filtration chromatography was 120 kDa (data not shown), indicating that the purified enzyme was dimeric. After incubating the purified preparation with AEH and NAD\(^+\), AEH in the reaction mixture decreased while AMH, ADH, and acetate increased, indicating that the purified enzyme converted the hydrazone to relevant hydrazides. Reactions without NAD\(^+\) resulted in a minimal decrease of AEH and VEH. The specific activity of the purified Hdh for AEH, AMH, and VEH was 16.7-, 14.5-, and 22.7- \( \mu \)mol min\(^{-1}\)mg\(^{-1}\), respectively, when 5 mM each of NAD\(^+\) and hydrazone were included.

**Stoichiometry of the Reaction**—We examined the stoichiometry of hydrazone consumption and hydrazide formation at 30°C in reactions containing 2 mM VEH, 2 mM NAD\(^+\) and 2 nmol of the enzyme in airtight tubes. After 120 min, 1.5 ± 0.1 mM of VEH were consumed and 1.5 ± 0.1, 1.5 ± 0.1, and 1.3 ±0.1 mM of VH, acetate and NADH, respectively, were formed. Levels of acetaldehyde were below the limits of detection. The results are consistent with the stoichiometry of the following reactions:

\[
\text{VEH + NAD}^+ + 2\text{H}_2\text{O} \rightarrow \text{VH + acetate + NADH + H}^+\]

This can be described more simply by focusing on the reacting functional groups as scheme 4 in Fig. 1B. We further confirmed the stoichiometry using AEH as a substrate (data not shown). Reactions without NAD\(^+\) resulted in a minimal decrease of AEH and VEH (data not shown). These results indicated that the purified enzyme was hydrazone-NAD\(^+\) dehydrogenase (Hdh).

**C. palmioleophila Hdh is a novel member of the ALDH superfamily**—We determined the amino terminal sequences of tryptic fragments of the purified protein, and cloned the cDNA and the gene for Hdh as described in EXPERIMENTAL PROCEDURES. The deduced amino acid sequence comprised of 519 amino acid residues and contained the internal amino acid sequences from the purified proteins (Fig. S1). The N-terminal amino acid sequence of purified enzyme started from Tyr 22, indicating that a limited proteolysis removed the N-terminal 21 residues in the purified enzyme. The calculated \( M_r \) (without the N-terminal residues) was 53,443, which agreed closely with that of purified enzyme (59,000) estimated by SDS-PAGE.

The deduced amino acid sequences of these proteins were significantly homologous to those of proteins in the Aldh superfamily and contained the sequence FYNTGEVC (Fig. S1) that is consistent with the motif for the catalytic Cys (Cys\(^{301}\)) and Gly (Gly\(^{308}\)) residues, which are strictly conserved in proteins of the Aldh superfamily (22). The sequence also contained VAFTGSTATG, which agrees with the consensus sequence around the conserved NAD-binding turn of the Rossmann fold (23). Conserved residues for NAD\(^+\) binding, Glu\(^{244}\) and Phe\(^{400}\) (22), and for the general base (Glu\(^{267}\)) were present in Hdh. Hempel et al. classified the Aldh superfamily of proteins into 13 families (22). Phylogenetic analysis showed that Hdh belongs to the fungal Aldh family in the Class 1/2 trunk (22, Fig. 4A). A database search uncovered that Hdh was related to putative gene product of *Pichia stipitis* and *C. albicans* with homologies of 77%, and 70%, respectively. Among the proteins with known functions, Ald4p and Ald5p from *Saccharomyces cerevisiae*, which oxidize acetaldehyde, were the most comparable to Hdh with 62% similarity.

**Characteristics of Hdh**—The purified enzyme required NAD\(^+\) or NADP\(^+\) for catalysis (Table 2). Purified Hdh was active over a pH range of 6.0 - 11.0, with an optimum at pH 9.0 (Fig. S2A). None of VH (5 mM), ADH (5 mM), NADH (0.1 mM) and acetate (3 mM) inhibited the reactions. When the enzyme was incubated with 0.5 mM acetate, 0.15 mM NADH and 0.5 mM ADH, neither NADH nor ADH decreased, indicating that the reaction was irreversible. The optimal temperature for enzymatic activity was 50°C (Fig. S2B). Most metal ions and chelating reagents (EDTA and 1,10-phenanthroline) minimally
affected the activity (91 to 110% relative to the mock control) except for Ni²⁺, Cu²⁺ and Hg²⁺. We routinely added 1 mM DTT to the reaction. In the absence of DTT, 62% of the Hdh activity was lost. These results together with powerful inhibition by Hg²⁺, and thiolate reagents (p-chloromercuribenzoate (pCMB) and iodoacetamide) (Table S2) suggested that the thiolate moiety is critical for expressing Hdh activity. No acetyl-CoA was produced in the reaction in the presence of both NAD⁺ and CoA, showing that Hdh has little CoA acylating activity (data not shown).

Adipic acid bis-(2-acetyl hydrazine) (AAH) and varelic acid 2-acetyl hydrazine (VAH) (Fig. 1C) are two-electron oxidized derivatives of AEH and VEH, respectively, that we applied to Hdh assays (Method A and B). After incubation with up to 5 mM AAH and VAH for over 2 h, AAH and VAH decreased very little, indicating that Hdh catalyzed neither of them (data not shown). This is consistent with our observation that C. palmioleophila MK883 could not grow when AAH was the sole source of carbon and that AAH was undetectable in the enzyme assay using AEH as a substrate. The substrate analog, ethyl isocyanoacetate (CH₃CH₂OC(O)CH₂N=C), which also has a functional group containing a C=N double bond as well as hydrazone, was not a substrate for Hdh (data not shown).

Initial Velocity Studies—The initial velocity of Hdh reaction was determined at variable concentrations of AEH with a fixed concentration (1.0 mM) of NAD⁺ or NADP⁺. Table 2 shows the apparent kinetic constants obtained by double reciprocal analysis. Under these conditions, ADH was detectable after the reaction (data not shown), indicating that the primary product of the enzyme reaction (AMH) is a substrate of Hdh. This suggested that the kinetic constants for the reaction with AEH are apparent. Steady-state kinetics showed that the reaction of VEH dehydrogenation followed a sequential mechanism (Fig. 5), indicating that the enzyme forms a ternary complex with VEH and NAD⁺

for VEH (Kᵦ) and NAD⁺ (Kᵦ) were 8.5 and 195 μM, respectively. The dissociation constants of VEH (Kᵦ) and NAD⁺ (Kᵦ) were 14.3 and 328 μM, respectively. Reaction with VEH and NADP⁺ resulted in similar plots (Fig. 5B) with substantially lower k_cat and higher Kᵦ and Kᵦ, indicating that the purified Hdh preferred NAD⁺ to NADP⁺.

Hdh hydrolyzed p-nitrophenyl (pNP) acetate in the absence of NAD⁺. We determined the kinetic constants for pNP acetate hydrolysis (Table 2). The k_cat for pNP acetate hydrolysis was 13.8 min⁻¹ and 4.0% and 6.5% of those for the NAD⁺- and NADP⁺- dependent dehydrogenation of VEH (Table 2). The Kₐ for pNP acetate (2.22 mM) was larger than that for VEH and the apparent Kₐ for AEH. The initial velocity of pNP hydrolysis in the presence of VEH was measured with varying concentrations of pNP acetate. The double reciprocal plots followed the kinetics of a competitive inhibition mechanism with an inhibition constant (Kᵦ) of 3.1 mM (Fig. 6A). The addition of NAD⁺ also decreased the rate of hydrolysis of pNP acetate and the double reciprocal plot followed the kinetics of a mixed inhibition mechanism with an inhibition constant (Kᵦ) of 0.18 mM (Fig. 6B). These results suggested that VEH and NAD⁺ can form respective binary complexes with Hdh, and that VEH, NAD⁺ and pNP acetate share at least in part, the same binding site on the enzyme.

The order of VEH and NAD(P)⁺ binding is intriguing since proteins in Aldh superfamily often bind substrates in an ordered manner. However, product inhibition studies (24), which are often used to determine binding order, were not feasible for studying the reaction catalyzed by Hdh since the reaction is irreversible and product inhibition did not occur. Instead, the results of our inhibition studies (Fig. 6) showed that VEH inhibited the esterase reaction less efficiently at Kᵦ = 3.1 mM, which is over 300-fold greater than the Kₐ of VEH for the dehydrogenase reaction. By contrast, NAD⁺ inhibited the esterase reaction at Kᵦ = 0.18 mM, which was in the same magnitude as the Kₐ for the dehydrogenase reaction. These results are
consistent with the high affinity of NAD$^+$ and low affinity of VEH for the free enzyme, which is reported in some Aldh (25).

Site Directed Mutagenesis— We produced and purified recombinant Hdh protein (rHdh) as 6× His tagged enzymes using the E. coli expression system. Recombinant Hdh migrated slightly later than the native Hdh in SDS-PAGE due to the 6× His residues on the N terminus (Fig. 3). The $K_m$ values for VEH and NAD$^+$ were 19.5 ± 3.6 and 247 ± 13 μM, which were similar to those of the native Hdh. The $k_{cat}$ for VEH oxidation by rHdh was 1260 ± 66 min$^{-1}$, which was 3.4-fold higher than that by native Hdh (Tables 2, 3). To obtain insight into the catalytic mechanism of Hdh, the conserved Cys$^{301}$ and Glu$^{267}$ residues were substituted with Ser and Ala by site-directed mutagenesis to construct C301S and E267A mutants, respectively (Fig. 3). The C301S mutant had <0.02% of the Hdh activity of wild type rHdh as well as Aldh activity, indicating that the thiolate moiety of Cys$^{301}$ is critical for Hdh activity. This is consistent with our observation that Hdh activity was sensitive to pCMB and requires DTT for maximal activity (Table S2).

The E267A mutants were active for oxidizing VEH and acetaldehyde and specific activities were 42 and 58 nmol min$^{-1}$ mg$^{-1}$, which were 0.4% and 0.8% of those of recombinantly expressed native Hdh when 1 mM NAD$^+$, and 0.5 mM VEH or 0.025 mM acetaldehyde were the substrates. Recombinant Hdh was active against pNP acetate with a $K_m$ of 720 ± 370 μM and a $k_{cat}$ of 197 ± 30 min$^{-1}$, confirming that the hydrolyzing activity of pNP acetate was associated with Hdh. Levels of pNP acetate hydrolysis by the C301S and E267A mutants were <0.01% and 0.03% of those by the wild type Hdh, showing that these residues are also critical for esterase activity.

Functional relation between Hdh and Aldh— The deduced amino acid sequence as well as the effect of mutations in Hdh resembled those of Aldh (26, 27). We therefore compared the Aldh activities of Hdh and S. cerevisiae Ald4p. The results showed that the $k_{cat}$ and $K_m$ values for both reactions were comparable (Table 3), indicating that Hdh catalyzes the Aldh reaction as efficiently as Ald4p. The activity of Ald4p is thought to be stimulated in the presence of potassium ions (28). Although we routinely added potassium ions to the reaction buffer, replacing it with sodium in the Hdh assay resulted in a loss of 85% of the activity (data not shown), indicating that Hdh was activated by potassium ions like Aldh. The preference of both nucleotide cofactors (NAD$^+$ and NADP$^+$) is characteristic of S. cerevisiae Ald4p (28) and Hdh (Table 3). These results implied that Hdh functioned as Aldh and with similar catalytic properties to Aldh, especially to Ald4p.

We measured the Hdh activity of Ald4p by using VEH as a substrate under the same conditions as the Hdh assay. The results showed that Ald4p expressed considerable activity for VEH, and that the reaction product was VH, indicating that Ald4p catalyzes the same reaction as Hdh. The $K_m$ for VEH was 37.8 ± 4.7 μM, which was comparable to that for acetaldehyde as well as that of rHdh for VEH and acetaldehyde (Table 3). The $k_{cat}$ for the reaction was as high as that for the oxidation of acetaldehyde, indicating that Ald4p oxidized VEH as efficiently as acetaldehyde. This is the first evidence that proteins in Aldh superfamily act on the C=N double bond. The Hdh reaction (Fig. 1, scheme 4) notably adds an additional hydration step to the Aldh reaction that requires only one (Fig. 4B, C, see Discussion).

Regulation and localization— When C. palmioleophila MK883 was cultured in MMAEH medium and then incubated with AEH for 120 min, 2.1 ± 0.2 mM of AEH was consumed and stoichiometric amounts of AMH (1.2 ± 0.2 mM) and ADH (0.9 ± 0.2 mM) were formed (Fig. 7A). A similar experiment using VEH resulted in the stoichiometric conversion of VEH to VH (data not shown). By contrast, yeast cells grown in glucose, succinate, or glucose plus AEH exhibited little AEH degradation. These results demonstrated that C. palmioleophila MK883 induced and repressed Hdh production in the presence of AEH and glucose, respectively.
This was essentially the same for the Hdh activity in cell-free extracts (Fig. 7B) and for transcription of the Hdh gene (Fig. 7C). These results indicated that the metabolic mechanisms for hydrazone assimilation are inducible and under the control of carbon catabolite repression at transcription level. These results, together with our findings that a preference for carbon sources was consistent with the substrate specificity of the purified enzyme, and that the enzyme recognizes specific hydrazones, suggested that Hdh is responsible for hydrazone assimilation by *C. palmioleophila* MK883.

We investigated the subcellular localization of Hdh since Ald4p and Ald5p of *S. cerevisiae* are located in the mitochondria (29, 30). Most Hdh activity co-fractionated with the soluble fractions containing cytosolic glucose-6-phosphate dehydrogenase in cell-free extracts of *C. palmioleophila* MK883 fractionated by differential centrifugation. Little Hdh activity was detected in the mitochondrial fractions (Fig. 7D), demonstrating the cytosolic localization of Hdh. Our results showed that the predicted amino acid sequence of Hdh has an extension on the N terminus that is missed in the purified enzyme (Fig. S1). This sequence exhibited lower homology with the N termini of Ald4p and Ald5p, and lacked the positively charged amino acid residues found in the typical mitochondrial targeting sequences. The extension was thought to be proteolytically cleaved in the cytosol of the yeast or during the purification procedures.

**Distribution of microorganisms that degrade hydrazone**— We examined the ability of 17 fungal strains to assimilate AEH. The results showed that *C. palmioleophila* NBRC10761, *C. colliculosa* JCM2199, *Pichia pastoris* X-33 and *Aspergillus nidulans* FGSC26 were positive, whereas *Aspergillus oryzae* RIB40, *Citeromyces matritensis* JCM2333, *Clavispora lusitaniae* JCM1814, *Fellomyces fuzhouensis* JCM7367, *Fusarium oxysporum* JCM11502, *F. solani* NBRC9425, *Hanseniaspora guilliermondii* JCM2200, *Kazachstania exigua* JCM1790, *Saccharomyces cerevisiae* BY4741, *S. selenospora* JCM7616, *Sterigmomyces elviae* JCM1822, *Trigonopsis variabilis* JCM1823, and *Williopsis saturnus* JCM3595 were negative. Among the 7 isolated AEH-assimilating strains, 2 (and strain MK883) exhibited the morphology of yeasts and 4, that of bacteria. Nucleotide sequences of the genes encoding 18S rRNA of the two yeasts were similar to those of *C. palmioleophila* NBRC10761 and of *Williopsis saturnus* with 97% and 98% identity. The 16S rRNAs of the bacteria were similar to those of *Pseudomonas putida*, *P. aeruginosa*, *Bacillus flexus* and *Delftia acidovorans*. These results implied that hydrazone-assimilating microorganisms are distributed among both Gram positive and negative bacteria, as well as among fungi, especially ascomycotina.

**DISCUSSION**

We discovered and isolated microorganisms that assimilate hydrazones, and purified the responsible enzyme. Microorganisms are believed to utilize vast numbers of chemicals and help to maintain balance in the global carbon and nitrogen cycle. Although many natural and artificial hydrazone compounds have been identified, little is known about microorganisms that thrive by metabolizing them. The present study is significant because we discovered a novel role of microorganisms in hydrazone degradation.

We showed that Hdh oxidizes hydrazones (AEH and VEH) to liberate corresponding hydrazines (ADH (or AMH) or VH) and acetate. A yeast cultured with AEH or VEH as the sole source of carbon accumulated stoichiometric amounts of ADH (and AMH) or VH, which is consistent with the inability of the yeast to assimilate these compounds. Meanwhile, the other product of the Hdh reaction (acetate) was undetectable during the culture period, indicating assimilation by the yeast. Common fungi activate acetate via acetyl-CoA synthetase to form acetyl-CoA in the first step of the acetate assimilation pathway (31). *C. palmioleophila* MK883 probably assimilates acetate via acetyl-CoA synthetase and assimilates hydrazones since
the strain can assimilate acetate and the purified Hdh itself lacks the CoA-acylating activity. These observations led to the explanation of the fungal mechanism of hydrazone assimilation described in Fig. 7E. The initial step was two-electron oxidation and hydration of the hydrazones through which the C=N double bond was cleaved. This step was catalyzed by the single enzyme Hdh purified in this study. The second step is presumed to be dependent upon acetyl-CoA synthetase. The formed acetyl-CoA is used as a source of both carbon and energy. We are confident that the key enzyme in this pathway is Hdh. We found that its production was inducible by the substrate and under the control of carbon catabolite repression in which large numbers of enzymes involved in carbon assimilation mechanisms are repressed in the presence of glucose (32). These results indicated that hydrazone assimilation is an adaptive mechanism that allows *C. palmioleophila* MK883 to survive under carbon-limited conditions.

Hydrazones can be chemically hydrolyzed to relevant hydrazines and carbonyl compounds under acidic conditions (Fig. 1B, scheme 3) (33). For example, VEH was hydrolyzed into VH and acetaldehyde at low pH (data not shown). Nevertheless, the biological system metabolizes hydrazones via the more complex NAD+-dependent oxidation reaction and not by simple hydrolysis (Fig. 1B, scheme 4). This is a remarkable difference between the chemical and biological systems for degrading hydrazones. We used hydrazones derived from acetaldehyde, which is highly reactive against biological compounds and cytotoxic at high concentrations. Presumably, the yeast oxidizes the acetaldehyde hydrazones using Hdh and produces less harmful acetate to avoid damage by acetaldehyde.

We are the first to isolate an enzyme that is involved in the oxidative cleavage of hydrazone bonds, although artificial activities of enzymes that attack hydrazones have been described. Rat liver glutamine transaminase (EC2.6.1.15) (34) physiologically transaminates the α-amino group of the glutamyl moiety to α-keto acids, and acts upon the γ-glutamyl hydrazones of the α-keto acids (11). This reaction is similar in that it cleaves the C=N bond of the substrate hydrazone, but the mechanism must be distinguished from that for Hdh in that it is accompanied by transfer of an α-amino group to the carbon atom of the hydrazone group. Another example of enzymes attacking hydrazones is peptidylglycine hydroxylase (EC1.14.17.3), which oxidizes carbon atoms on hydrazone like Hdh (10). However, it uses dioxygen as the oxidant, which is in sharp contrast to Hdh. Furthermore, the reaction product of peptidylglycine hydroxylase is a relevant acyl hydrazide. This indicates that the peptidylglycine hydroxylase lacks the hydrolyzing activity of hydrazides and thus cannot cleave the carbon-nitrogen bond of hydrazide, which is in contrast to the Hdh reaction that involves both oxidizing and hydrolyzing mechanisms.

Enzymes that attack C=N bonds other than hydrazones are more ubiquitous. Imine-degrading enzymes such as arginine deiminase (EC3.5.3.6) and agmatine deiminase (EC3.5.3.12) hydrolyze (deiminize) the C=NH₂ bond of imino groups to form ammonium (35, 36). Oximes (RC=N-OH) are dehydrated to form nitrile by distinct bacteria and assimilated (37). These reactions are based on hydrolysis or dehydration mechanisms and are in contrast to Hdh, which involves the redox reaction. The naturally occurring C=N compound Δ-1-pyrroline-5-carboxylate (P5C) is an intermediate of proline synthesis and degradation (38). P5C dehydrogenases (Pcdh) (EC1.5.1.12) (39, 40) catalyze the NAD⁺-dependent oxidation of P5C and belong to the Aldh superfamily (22, 41) like Hdh. However, the initial step of the reaction is the non-enzymatic hydrolysis of the C=N bond of P5C to form glutamate-γ-semialdehyde, which lacks a C=N bond and its semialdehyde moiety is oxidized to acid (that is, the Aldh reaction). By contrast, we showed that Hdh oxidized and hydrated C=N compounds in a single catalytic cycle (Fig. 1B, scheme 4, see below). These results indicated that the isolated Hdh is distinguishable from known enzymes in terms of its substrates (hydrazones) and physiological function.
(hydrazone assimilation).

Besides the unique catalytic reaction, Hdh belongs to the Aldh superfamily and notably shares common features with Aldh. Like Hdh, dimeric Aldh comprising 40 to 60 kDa monomers are prevalent (22, 41). Some Aldh exhibits esterase activity against pNP acetate as well as Hdh (40). Furthermore, the isolated enzyme exhibited Aldh, as well as Hdh activity. Both activities were dramatically decreased by a mutation of the Cys residue conserved among proteins in the Aldh superfamily or by thiolate reagents. These observations indicate that Hdh oxidizes hydrazones by a similar mechanism to Aldh, which uses the thiolate moiety of the Cys as a nucleophile that attacks the carbonyl carbon of aldehydes. This generates a thioacyl intermediate that is then hydrated to form an acid (42) (Fig. 4B). By analogy, the thiolate moiety of Cys^301 in Hdh attacks the carbon atom of the C=N bond upon hydrazone oxidation. This forms thiohemiacetal, followed by hydride transfer to NAD^+, and generates an intermediate (I_1) that will be hydrated (Fig. 4C). We showed that AAH and VAH, which are two-electron oxidized compounds of relevant hydrazones, are not substrates for the Hdh reaction, indicating that the I_1-generating step is intimately coupled to hydrazone oxidation. Another possibility is that the enzyme transfers a water molecule to the substrate (or an intermediate) and then oxidizes it. However, this is less likely since the chemical hydration of hydrazones easily cleaves the C=N bond and liberates hydrazide and aldehyde. The subsequent hydration step of I_1 by Hdh is unique. Hydration via Aldh is thought to proceed at the carbon atom of the acyl-enzyme intermediate and the reaction cycle is completed by liberating acid and thiolate (Fig. 4B). If Hdh hydrates I_1 to liberate a thiolate enzyme like Aldh, it must produce acid hydrazides such as AAH and VAH, which are then hydrolyzed to complete the catalytic cycle. However, this is less likely since we detected little active Hdh hydrolysis of these acid hydrazides. Rather, the hydration reaction resulted in cleavage of the C=N bond and consequent hydrazide (ADH and VH) production, while the thioester bond of I_1 remained intact (Fig. 4C). The resulting intermediate (I_2) is exactly the same as the intermediate for the Aldh reaction (Fig. 4B), and it is probably hydrated by the second water molecule, deacylated, and then it can complete the catalytic cycle. The ‘oxidative hydrolysis’ reaction is the most unique feature of Hdh among known enzymes.

The proposed roles of general bases in the Aldh reaction are activating the conserved Cys to generate thiohemiacetal, and activating water to deacylate the enzyme (Fig. 4B) (26). A glutamate residue might function in both or either of the steps in Aldh (26). Here we showed that the E267A mutant of Hdh possessed only 0.4% of the normal level of Hdh, suggesting that Glu^267 functions as the general base. As discussed above, the kinetic mechanism of Hdh involved the activation of two water molecules. The potential for the corresponding Glu in Aldh to activate water to attack the thioacyl intermediate (43) suggests that Glu^267 of Hdh is at least involved in the second step in which water hydrates I_2. The role of Glu^267 in activating the first water molecule remains to be studied. Our observations imply that Hdh finely tunes these catalytic steps by facilitating the general bases for the oxidative hydrolysis of hydrazones in a more complex manner than in the Aldh reaction.

The Aldh superfamily consists of >500 predicted proteins that are widely distributed from bacteria to higher eukaryotes (22). Among them, proteins with known catalytic functions can oxidize carbonyl carbon (22). Otherwise, the catalytic functions of most predicted Aldh proteins found in databases are obscure, and whether they react with C=N compounds remains unknown. Thus our findings that Hdh and Ald4p function as Hdh as well as Aldh suggest their potential for catalyzing C=N compounds. This study also showed that hydrazone-assimilating microorganisms are widely distributed across phyla. Our findings shed light on the hitherto undiscovered biology of hydrazone and C=N compounds.
REFERENCES


**FOOTNOTES**

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The abbreviations used are: AAH, adipic acid bis-(2-acetyl hydrazine); ADH, adipic acid dihydrazide; AEH, adipic acid bis-(ethylidene hydrazide); Aldh, aldehyde dehydrogenase; AMH, adipic acid ethylidene hydrazide; DTT, dithiothreitol; Hdh, hydrazone dehydrogenase; VAH, varelic acid acetyl hydrazine; VEH, varelic acid ethylidene hydrazide; VH, varelic acid hydrazide; Δ-1-Pyrroline-5-carboxylate, P5C; Pcdh, Δ-1-pyrroline-5-carboxylate dehydrogenases; pCMB, p-chloromercuribenzoate; SDS-PAGE, sodium dodecyl sulfate- polyacrylamide gel electrophoresis
FIGURE LEGENDS

FIGURE 1. Hydrazones and their derivatives used in this study.
A. Synthesis of hydrazones. Adipic acid dihydrazide (ADH, (1)) reacts with one molecule of acetaldehyde to produce adipic acid ethyldene hydrazide (AMH, (2)), which forms adipic acid bis-(ethyldene hydrazide) (AEH, (3)) by reaction with additional acetaldehyde (Scheme 1). Similar reaction between varelic acid hydrazide (VH, (4)) and acetaldehyde forms varelic acid ethyldene hydrazide (VEH, (5)) (Scheme 2). B. General description of hydrolysis reaction of acetaldehyde hydrazone that forms hydrazide and acetaldehyde (Scheme 3), which is in contrast to oxidative degradation of hydrazone revealed in this study (Scheme 4). C. Structures of adipic acid bis-(2-acetyl hydrazine) (AAH, (6)) and varelic acid acetyl hydrazine (VAH, (7)) synthesized herein.

FIGURE 2. Time-dependent degradation of AEH by C. palmioleophila MK883.
A, C. palmioleophila MK883 was cultured in MM medium containing 42 mM AEH at 30°C, and concentrations of AEH (closed circles), AMH (triangles), ADH (squares), and optical density (A$_{600}$) of culture broth (open circles) were measured as described in EXPERIMENTAL PROCEDURES. Open squares, concentrations of AEH in control experiment without cells. B. Similar experiments proceeded in MM medium containing 10 mM VEH. Symbols: closed circles, VEH; squares, VDH; open circles, optical density (A$_{600}$) of culture broth.

FIGURE 3. SDS-PAGE of purified enzyme.
Purified enzymes (1 μg) were resolved by SDS-PAGE on 10% polyacrylamide gels and stained with Coomassie brilliant blue. Lanes: 1, markers (Bio-Rad Precision Protein Standard kit); 2, native enzyme; 3, rHdh; 4, C301S mutant; 5, E267A mutant.

FIGURE 4. Phylogenetic tree and catalytic mechanism of Hdh/Aldh.
A. Phylogenetic tree of predicted proteins in fungal Aldh family. Abbreviations; Ani, Aspergillus nidulans, Anig, A. niger; Aor, A. oryzae; Cal, C. albicans; Cpa, C. palmioleophila; Lel, Lodderomyces elongisporus; Ncr, Neurospora crassa; Pan, Pichia angusta; Pst, P. stipitis; Sce, S. cerevisiae; Spo, Schizosaccharomyces pombe. The outgroup comprised Put2 proteins belonging to γ-glutamate semialdehyde dehydrogenase family. Accession numbers are shown in parenthesis. B and C. Catalytic mechanisms of aldehyde oxidation by Aldh (B) and of oxidative hydrolysis of hydrazones by Hdh (C) and. ‘B’ represents unidentified general base.

FIGURE 5. Double- reciprocal plots of initial rates of reduction of NAD(P)$^\text{+}$.
Steady-state turnover of hydrazone dehydrogenase activity was measured using Method B. A. Concentrations of VEH were varied at fixed concentrations of NAD$^+$ equal to 0.075 mM (closed circles), 0.15 mM (triangles), 0.3 mM (closed squares), 0.6 mM (open circles), 1.5 mM (open squares). B. Concentrations of VEH were varied at fixed concentration of NADP$^+$ equal to 0.16 mM (closed circles), 0.33 mM (triangles), 0.65 mM (closed squares), 1.3 mM (open circles), 2.6 mM (open squares).

FIGURE 6. Inhibition of pNP acetate hydrolysis by VEH and NAD$^+$.
Steady-state turnover of pNP acetate hydrolase activity was measured. A. Initial velocity was determined as function of pNP acetate concentration in absence of VEH (circles) and at concentrations of VEH equal to 1 mM (triangles), 3 mM (squares), 6 mM (diamonds) and 12 mM (open circles). B. Initial velocity was determined as a function of pNP acetate concentration without NAD$^+$ (circles) and at concentrations of NAD$^+$ equal to 0.2 mM (triangles), 0.6 mM (squares) and 1.0
mM (open circles).

FIGURE 7. Regulation and intracellular localization of Hdh.
A. C. palmioleophila MK883 was cultured in MM medium containing 30 mM AEH (circles), 200 mM glucose (diamonds) and both 30 mM AEH and 200 mM glucose (triangles) at 30°C for 10 h. Harvested cells ($A_{600} = 0.9$) were incubated at 30°C in 50 mM potassium phosphate (pH 7.2) containing 4.5 mM AEH. Control reaction without cells is represented by squares. Concentrations of AEH (closed symbols), AMH (open symbols) and ADH (closed symbols with dotted line) were measured. B. Hydrazone-degrading activity in cell-free extracts. C. palmioleophila MK883 was cultured as described above, and then AEH degradation was assayed in cell-free extracts (Method A). C. Quantitative PCR analysis of Hdh gene transcript. Bars indicate values reported as relative expression rate obtained by RT-PCR and normalized to 18S rRNA. D. Cytosolic localization of hydrazone dehydrogenase activity. Absolute activity for 100% was 49-, 22-, and 226- nmol min$^{-1}$ mg$^{-1}$ for AEH dehydrogenase (closed bars), glucose-6-phosphate dehydrogenase (shaded bars), and cytochrome c oxidase (open bars). Standard errors in panels A to D are below 20%. E. Model for hydrazone assimilation by C. palmioleophila MK883.
<table>
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<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (µmol min⁻¹)</th>
<th>Specific activity (µmol min⁻¹mg⁻¹)</th>
<th>Yield (%)</th>
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<td>Cell-free extract</td>
<td>296</td>
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<tr>
<td>DEAE cellulose</td>
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### TABLE 2
Kinetic constants of *C. palmioleophila* MK883 Hdh for various substrates

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<tr>
<th>Variable substrate&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fixed substrate&lt;sup&gt;b&lt;/sup&gt;</th>
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<th>$K_B$</th>
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<tr>
<td></td>
<td>μM</td>
<td>μM</td>
<td>min&lt;sup&gt;-1&lt;/sup&gt;</td>
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<tr>
<td>AEH</td>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>20.5 ± 5.8</td>
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<td>408 ± 32</td>
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<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt;</td>
<td>AEH</td>
<td>283 ± 68</td>
<td>-</td>
<td>308 ± 20</td>
<td>1.09</td>
<td>-</td>
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<tr>
<td>NADP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>AEH</td>
<td>401 ± 128</td>
<td>-</td>
<td>164 ± 16</td>
<td>0.41</td>
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<tr>
<td>VEH, NAD&lt;sup&gt;+&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>8.5 ± 1.3</td>
<td>195 ± 23</td>
<td>341 ± 12</td>
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<td>VEH, NADP&lt;sup&gt;+&lt;/sup&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>9.8 ± 1.5</td>
<td>697 ± 79</td>
<td>212 ± 9.3</td>
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<td>pNP acetate</td>
<td>-</td>
<td>2215 ± 322</td>
<td>-</td>
<td>13.8 ± 0.83</td>
<td>0.00623</td>
<td>-</td>
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Enzyme activity was determined by measuring increases in absorbance at 340 nm (Method A). Data are means of three experiments.

<sup>a</sup> Little (< 3 x 10<sup>-2</sup> μmol min<sup>-1</sup> mg<sup>-1</sup>) activity was detected by using AAH, VAH, ADH and ethyl thiocyanatoacetate (up to 5 mM) as substrates.

<sup>b</sup> Activity was measured with 1 mM of each substrate (inhibitor).

<sup>c</sup> Dissociation constants: $K_{iA} = 14.3 ± 3.8$ μM, $K_{iB} = 328 ± 67$ μM.

<sup>d</sup> Dissociation constants: $K_{iA} = 9.8 ± 2.7$ μM, $K_{iB} = 697 ± 141$ μM.
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<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/K_m$ (min⁻¹ μM⁻¹)</th>
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<td>acetaldehyde⁴</td>
<td>14.3 ± 4</td>
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<td>Ald4p</td>
<td>VEH</td>
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<td></td>
<td>acetaldehyde</td>
<td>16.9 ± 3.3</td>
<td>15.1 ± 0.97</td>
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</tbody>
</table>

⁴ Hydrazone and aldehyde dehydrogenase activity were measured with 1 mM NAD⁺.
FIGURE 1.
FIGURE 2.
FIGURE 3.
FIGURE 4.

A

B

C
FIGURE 5.
FIGURE 6.
FIGURE 7.

A

B

C

D

E

induction

repression

hydrazones

hydrazides

NAD+, 2H2O

NADH, H+

Hdh

HDH gene

glycine

CH₂COOH

Acs

CH₂CO-CoA

assimilation

CoA, ATP

AMP, PPI

H₂O

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SUPPLEMENTAL INFORMATION

Synthesis of hydrazones and their derivatives

1. Methods
Reagents and solvents for synthesis were commercial grade. 1H and 13C NMR spectra were recorded on an EX-270 NMR Spectrometer (JEOL, Japan) in dimethylsulfoxide (DMSO)-d6 with trimethylsilane (TMS) as the internal standard. 1H-COSY and HMBC spectra were recorded on an AVANCE-600 NMR Spectrometer (Bruker) in DMSO-d6 with TMS as the internal standard. The splitting profile of 1H NMR spectra comprised: s, singlet; d, doublet; t, triplet; q, quartet; qui, quintet; dd, double doublet; m, multiplet. ESI mass spectra were recorded on an LC/MS/MS mass spectrometer (API2000TM, Applied Biosystems) equipped with Capcellpak (Shiseido Co., Japan). The mobile phase was 90% methanol. The flow rate and temperature were maintained at 0.5 ml min−1 and 30°C, respectively.

2. Results
We synthesized hydrazones and acetic hydrazides (compounds (2), (3), (5), (6) and (7) in FIGURE 1.) as follows and the structures were established by 1H-NMR, 1H-COSY, 13C-NMR, HMBC and electron spray ionization mass (ESI) spectra. Some of the characteristic signals could be readily assigned in the one-dimensional 13C- and 1H- NMR spectra, but for resolving signals of AAH and VAH, two-dimensional techniques were required. A crosspeak of C-1' to NH-2 and H-2' in the HMBC spectrum of (6) appeared due to long-range coupling of 1H and 13C over the amide bonds and two acetyl carbons (data not shown). Similarly, the C-1 atom correlated with NH-1, H-2, and H-3 protons in the HMBC spectrum. These correlations were also observed in the HMBC spectrum of (7), confirming the assignments of signals to individual atoms. All compounds yielded a molecule ion [M + H]+ peak with the calculated value on ESI mass spectra. The gas chromatography-electron impact-mass spectrum of (6) closely matched the published spectrum (Ref. 1).

The 1H- and 13C-NMR spectra of (3) showed double signals of H-1’, NH, C1’, C1, C2, and C3, indicating that these compounds are mixtures of stereoisomers. They were probably E/Z isomers of the C=N bond, and/or stable conformers around the CO-NH group as described for similar compounds (Ref. 2). The double signals were similar for (5), and both contained C=N bonds, suggesting that (5) also comprises two stereoisomeromic forms.

2-1. Adipic acid ethyldiene hydrazide (AMH) (compound (2) in FIGURE 1.)
Acetaldehyde (30% in water, 1.2 molar excess) was added to adipic acid dihydrazide (0.3 g) in THF (2 ml) over 2 h on ice. The solution was dried in a hood, resuspended in water (10%), and separated by an HPLC system as described in EXPERIMENTAL PROCEDURES.

δH (DMSO-d6): 2.08 (2H, m, H-2), 1.50 (2H, m, H-3), 1.44 (2H, m, H-4), 1.99 (2H, m, H-5), 7.29, 7.44 (50%, 50%, 1H, q, H-1’), 1.83, 1.85 (50%, 50%, 3H, d, H-2’), 10.74, 10.88 (50%, 50%, 1H, d, N-1), 8.88 (1H, t, N-3), 4.12 (2H, d, N-4). ESI-MS: [M + H]+ m/z 201.2.

2-2. Adipic acid bis-(ethyldene hydrazide) (AEH) (compound (3) in FIGURE 1.)
A 6-fold molar excess of acetaldehyde (30% in water) was added to adipic acid dihydrazide (10 g) over 2 h on ice. Precipitates were collected, washed with water, and dried in a hood over night to yield the product.

δH (DMSO-d6): 2.07 (4H, br, H-2), 1.52 (4H, m, H-3), 7.28, 7.43 (50%, 50%, 2H, q, H-1’), 1.83, 1.85 (50%, 50%, 6H, d, H-2’). δC (DMSO-d6): 167.52, 173.10 (C-1), 31.46, 33.82 (C-2), 23.89, 24.80 (C-3), 142.78, 145.82 (C-1’), 18.14 (C-2’). ESI-MS: [M + H]+ m/z 227.2.
2-3. Varelic acid ethylidene hydrazide (VEH) (compound (5) in FIGURE 1.)

Acetaldehyde (30% in water, 1.2 molar excess) was added to pentanoic acid hydrazide (0.3 g) in THF (2 ml) over 2 h on ice. The solution was dried in a hood to yield the product.

\[ \delta \text{H (DMSO-}d_6\text{): } 2.08, 2.45 (50\%, 50\%; 2H, t, H-2), 1.50 (2H, qui, H-3), 1.28 (2H, m, H-4), 0.87 (3H, t, H-5), 7.28, 743 (50\%, 50\%; 1H, q, H-1'), 1.83, 1.85 (50\%, 50\%, 3H, d, H-2'), 10.72, 10.89 (50\%, 50\%; 1H, s, N-H). \]

\[ \delta \text{C (DMSO-}d_6\text{): } 167.89, 173.44 (C-1), 31.47, 33.73 (C-2), 26.40, 27.22 (C-3), 21.85, 21.96 (C-4), 13.77, 13.87 (C-5), 142.81, 145.94 (C-1'), 18.22 (C-2'). \]

ESI-MS: [M + H]^+ \text{ m/z 143.2.}

2-4. Adipic acid bis-(2-acetyl hydrazine) (AAH) (compound (6) in FIGURE 1.)

A 6-fold molar excess of acetic anhydride was added to adipic acid dihydrazide (0.3 g) in toluene (3 ml), refluxed on a water bath for 5 h, and then the mixture was cooled on ice. The resulting precipitate was collected, and dried in vacuo.

\[ \delta \text{H (DMSO-}d_6\text{): } 2.10 (4H, m, H-2), 1.51 (4H, m, H-3), 1.86 (6H, s, H-2'), 9.69 (2H, d, N-1), 9.64 (2H, d, N-2). \]

\[ \delta \text{C (DMSO-}d_6\text{): } 170.42 (C-1), 32.74 (C-2), 24.49 (C-3), 167.56 (C-1'), 20.33 (C-2'). \]

ESI-MS: [M + H]^+ \text{ m/z 259.2.}

2-5. Varelic acid acetyl hydrazine (VAH) (compound (7) in FIGURE 1.)

Compound (7) was prepared in essentially the same manner as compound (6) using pentanoic acid hydrazide (0.3 g) and acetic anhydride (3 molar excess).

\[ \delta \text{H (DMSO-}d_6\text{): } 2.12 (2H, t, H-2), 1.48 (2H, qui, H-3), 1.28 (2H, m, H-4), 0.86 (3H, t, H-5), 1.74 (3H, s, H-2'), 9.66 (1H, s, N-1), 9.61 (1H, s, N-2). \]

\[ \delta \text{C (DMSO-}d_6\text{): } 170.76 (C-1), 32.80 (C-2), 27.18 (C-3), 21.67 (C-4), 13.72 (13.72), 167.69 (C-1'), 20.49 (C-2'). \]

ESI-MS: [M + H]^+ \text{ m/z 159.0.}
FIGURE LEGENDS

FIGURE S1. Multiple alignment of deduced amino acid sequences of Hdh and Aldh.
Residues that are 100% conserved between Hdh and Aldh are highlighted in blue with white letters. Residues conserved in 75% of sequences are shaded gray with black letters. Amino acid positions of Hdh are numbered from the 22th Tyr as 1st (Tyr1). Asterisks under sequences indicate amino acid residues noted in text. Gln267 and Cys301 were mutated in this study. Consensus segments of Aldh proposed by Hempel et al. are boxed. Dashed lines indicate amino terminal sequences of tryptic fragments of Hdh. Accession numbers of sequences are AB361432 (Hdh), YOR374W (S. cerevisiae Ald4p), YER073W (S. cerevisiae Ald5p), and NP058968 (Rattus norvegicus Aldh).

FIGURE S2. Effects of pH and temperature on AEH dehydrogenase activity.
A. Reactions were incubated for 120 min at 30°C (Method A in EXPERIMENTAL PROCEDURES) in: citrate-phosphate (4.0-6.0; squares) (Ref. 3), 50 mM potassium phosphate (6.0-7.5; diamonds), 50 mM Bicine (7.5-9.0; circles) and 100 mM glycine/NaOH (9.0-11.0; triangles). B. Reactions proceeded for 120 min at various temperatures (Method A in EXPERIMENTAL PROCEDURES). Relative activity is expressed as ratio (%) of maximal activity under our experimental conditions.

REFERENCES

**TABLE S1**

**Oligonucleotide primers used in this study**

FA, FB, FC, and FD were synthesized based on the internal amino acid sequences (MCSRGDV, PMVPFG, QIIPWNF, and YNDFNPM) of the Hdh. Mutated residue is underlined.

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\(^a\) Hdh activity for AEH was measured using \textit{Method A} and hydrolyzing activity for pNP acetate was measured in absence of NAD\(^+\) after the enzyme (1 \(\mu\)g) was incubated with each reagent in 50 mM potassium phosphate (pH 7.2) for 1 h at room temperature. Final concentration of the compounds was 1 mM unless otherwise stated. Data are means of three experiments and standard errors are below 5%.

\(^b\) Methanol was added to final concentration of 0.5\%(v/v) to increase solubility.

\(^c\) Final concentrations were 0.1%.
FIGURE S2.
Novel dehydrogenase catalyzes oxidative hydrolysis of carbon-nitrogen double bonds for hydrazone degradation

Hideomi Itoh, Tetsuya Suzuta, Takayuki Hoshino and Naoki Takaya

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