A Histidine Residue in the Catalytic Mechanism Distinguishes *Vibrio harveyi* Aldehyde Dehydrogenase from Other Members of the Aldehyde Dehydrogenase Superfamily†

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ABSTRACT: Aldehyde dehydrogenases (ALDHs) catalyze the transfer to NAD(P) of a hydride ion from a thiohemiacetal derivative of the aldehyde coupled with a cysteine residue in the active site. In *Vibrio harveyi* aldehyde dehydrogenase (Vh-ALDH), a histidine residue (H450) is in proximity (3.8 Å) to the cysteine nucleophile (C289) and is thus capable of increasing its reactivity in sharp contrast to other ALDHs in which more distantly located glutamic acid residues are proposed to act as the general base. Mutation of H450 in Vh-ALDH to Gln and Asn resulted in loss of dehydrogenase, (thio)esterase, and acyl-CoA reductase activities; the residual activity of H450Q was higher than that of the H450N mutant in agreement with the capability of Gln but not Asn to partially replace the ϵ-imino group of H450. Coupled with a change in the rate-limiting step, these results indicate that H450 increases the reactivity of C289. Moreover, for the first time, the acylated enzyme intermediate could be directly monitored after reaction with [^{3}H]tetradecanoyl-CoA showing that the H450Q mutant was acylated more rapidly than the H450N mutant. Inactivation of the wild-type enzyme with *N*-ethylmaleimide was much more rapid than the H450Q mutant which in turn was faster than the H450N mutant, demonstrating directly that the nucleophilicity of C289 was affected by H450. As the glutamic acid residue implicated as the general base in promoting cysteine nucleophilicity in other ALDHs is conserved in Vh-ALDH, elucidation of why a histidine residue has evolved to assist in this function in Vh-ALDH will be important to understand the mechanism of ALDHs in general, as well as help delineate the specific roles of the active site glutamic acid residues.

The super or extended family of aldehyde dehydrogenases (ALDHs)[1] now contains well over 150 different proteins composed of polypeptides of approximately 50−55 kDa in molecular mass which are related in sequence (*1*). ALDHs catalyze the NAD(P)-dependent oxidation of a variety of aldehydes to the corresponding acids and are widespread in nature presumably due to the many different aldehyde metabolic intermediates and the need to maintain the reactive and thus potentially toxic endogenous or exogenous aldehydes at relatively low levels in the cell.

All ALDHs that have been characterized in the ALDH superfamily are homopolymers composed of two or four polypeptides of 50−55 kDa. Over the last three years, seven crystal structures of members of this family have been determined starting with rat ALDH3 (*2*) and extending to bovine liver ALDH2 from mitochondria (*3*), cod liver betaine ALDH9 (BALDH) (*4*), sheep cytosolic ALDH1 (*5*), *Streptococcus mutans* ALDH (*6*), mouse retinal ALDH (type II) (*7*), and *Vibrio harveyi* ALDH (Vh-ALDH) (*8*). Two of the crystallized enzymes are dimers (ALDH3 and Vh-ALDH) with the remaining ALDHs being tetramers. Both bacterial enzymes are NADP specific while the crystallized eukaryotic ALDHs are NAD specific. The location of the adenosine moiety appears to be reasonably well established in the structures with bound NAD(P), however, there is still controversy over the exact location of the nicotinamide moiety in the active site during catalysis as well as the identity and role of some of the amino acids believed to be directly involved in the catalytic mechanism.

The central feature of the active site of all the ALDHs is a Cys nucleophile which forms a thiohemiacetal derivative with the aldehyde before transfer of a hydride to NAD(P) to form an acylated intermediate that is hydrolyzed to the corresponding acid. Other polar residues conserved in the active site of the crystallized ALDHs are an Asn residue proposed to stabilize the thiohemiacetal derivative (*9*, *10*) and two Glu residues located between 5 and 9 Å from the Cys nucleophile. Depending on the specific ALDH, one or the other of the Glu residues has been proposed to function as a general base and assist in the deprotonation of the cysteine nucleophile (*10*, *11*). However due to the large

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[1] Abbreviations: ALDHs, aldehyde dehydrogenases; Vh-ALDH, V. harveyi aldehyde dehydrogenase; ALDH1, sheep cytosolic aldehyde dehydrogenase; ALDH2, bovine mitochondrial aldehyde dehydrogenase; ALDH3, rat tumor aldehyde dehydrogenase; ALDH9 or BALDH, cod liver betaine aldehyde dehydrogenase; GGALDH, γ-glutamyl semialdehyde dehydrogenase; SSALDH, succinate semialdehyde dehydrogenase; 2′,5′-ADP, adenosine 2′,5′-bisphosphate; LU, light units; ND1, delta imino nitrogen; NE2, epsilon imino nitrogen; NEM, *N*-ethyl maleimide; wt, wild-type.

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distance from the Cys nucleophile, the Glu residue must mediate its effect via a water molecule or another polar residue and/or be brought in proximity to the Cys nucleophile by a conformational change.

The recent elucidation of the structure of the NADP-specific ALDH (Vh-ALDH) from the luminescent bacterium, *V. harveyi*, provided the unexpected result that a His residue (H450) was in proximity to the Cys nucleophile (C289). This result was even more surprising since the Glu residues (E253 and E377 in Vh-ALDH) implicated as the general base in the catalytic mechanisms of other ALDHs were in the same relative positions in the active site of Vh-ALDH as found in the other crystallized ALDHs (*8*). As the proximity of H450 to the Cys nucleophile in Vh-ALDH implicates this residue in the catalytic reaction, investigation of the function of H450 in Vh-ALDH should provide a new perspective to the role specific amino acid residues play in the mechanism of ALDHs.

In the present paper, the effects of mutating H450 on the kinetic properties of Vh-ALDH and their relationship to the reactivity of the Cys nucleophile have been investigated. The results provide strong support for H450 functioning as a general base in the mechanism of Vh-ALDH and show that different residues have evolved for increasing the reactivity of the Cys nucleophile even though the basic architecture of the active site has been conserved.

MATERIALS AND METHODS

Materials. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Labs or Pharmacia. [^{35}S]-Methionine (800 Ci/mmol) and [^{35}S]dATP (1400 Ci/mmol) were obtained from Du Pont-New England Nuclear. NADP, NAD, and aldehydes were purchased from Sigma-Aldrich. [^{3}H]Tetradecanoyl-CoA (20 Ci/mmol) was prepared from [^{3}H]tetradecanoic acid (New England Nuclear) by the method of Bishop and Hajra (*12*) as described previously (*13*). The bacterial strains used in these studies were *Escherichia coli* K38 and *V. harveyi* B392.

Expression of Native Vh-ALDH and Mutant ALDHs Using the Bacteriophage T7 RNA Polymerase/Promoter System. E. coli K38 cells were transformed with the pT7-5 plasmid containing recombinant DNA and the pGP1-2 plasmid coding for T7 RNA polymerase under control of a temperature sensitive repressor (*14*). For expression of protein, cells were grown in Tabor's media with 50 μg/mL ampicillin and kanamycin at 30 °C up to $A_{590} = 1.5$, and the temperature shifted to 42 °C. Rifampicin was then added to a final concentration of 200 μg/mL to inhibit *E. coli* RNA polymerase. The cells were grown at 30 °C for another 60 min before harvesting by centrifugation.

Purification of Recombinant Wild-Type and Mutant ALDHs. Cells were resuspended in 50 mM sodium potassium phosphate, pH 7, 10 mM β-mercaptoethanol, and lysed by sonication at 4 °C. The lysate was centrifuged at 24000*g* in a Sorvall RC 5B for 20 min, and the supernatant diluted to 1−2 mg/mL of protein with the same buffer. Samples were applied to a column of adenosine 2′,5′-bisphosphate (2′,5′-ADP) linked to Sepharose (Pharmacia) equilibrated in 50 mM phosphate buffer, pH 7 at 4 °C. The column was washed with 10−50 times the column volume of 50 mM phosphate, pH 7, and eluted with 100 μM NADP in the same buffer. The eluted enzyme fractions were pooled and concentrated by dialysis against 30% glycerol, 50 mM sodium potassium phosphate, pH 7, and 10 mM β-mercaptoethanol at 4 °C. Enzymes were stored at −20 °C.

Protein Assays. Protein concentrations were determined using the Bio-Rad protein determination kit with bovine serum albumin as a standard.

Fluorescence Assays. ALDH activities were measured on a Hitachi F-3010 fluorometer in 50 mM sodium potassium phosphate, pH 8 at 25 °C. Production of NAD(P)H was monitored by measuring the fluorescence emission at 460 nm after excitation at 340 nm. The wavelength dispersion was 5 nm for both excitation and emission.

Enzyme Kinetics. All kinetic measurements were performed at least three times, and mean values were used for subsequent plots or calculations. Complete two-substrate kinetics were measured with heptanal and NAD(P). The reciprocal of the initial velocities were plotted versus the reciprocal of the concentration of the first substrate (e.g., NADP) at different fixed concentrations of the second substrate (e.g., aldehyde). The Michaelis constants and k_{cat} values were calculated from secondary plots of intercepts and slopes. All standard errors for the two-substrate kinetics were between 2 and 18% of the indicated values. Measurements with dodecanal as substrate were conducted at saturating aldehyde concentrations and the apparent K_m for NAD(P) and k_{cat} determined from a plot of the initial velocity versus the NAD(P) concentration using the computer program GraphFit to provide the best fits and standard errors.

Site-Directed Mutagenesis. The *V. harveyi* ALDH gene in M13 was mutated based on the method described by Kunkel (*15*) using the Muta-Gene M13 in Vitro Mutagenesis Kit from Bio-Rad. The codon for histidine at position 450 (CAC) was altered to CAA (Gln) and AAT (Asn). The mutated DNA was transferred to the pT7-5 vector, the sequence reconfirmed, and the mutated gene expressed in *E. coli* as described above.

DNA Sequencing. DNA sequencing was performed using the Sequenase DNA sequencing kit (version 2) from USB based on the dideoxy chain-termination method (*16*).

Acyl-CoA Reductase Assay. The reduction of tetradecanoyl-CoA to tetradecanal was measured from the luminescence response of luciferase to the aldehyde product as described by Byers and Meighen (*17*). After incubation of the enzyme with 5 μM tetradecanoyl-CoA in 1 mL of 0.05 M phosphate, pH 7, and the indicated concentration of NADPH (usually 1−10 μM), 5 μg of *P. phosphoreum* luciferase was added and the maximum luminescence intensity recorded in light units (LU) upon injection of 1 mL of 50 μM $FMNH_2$. Under the conditions of the assay, one LU corresponds to 3.8 pmol of tetradecanal.

Acyl-CoA Cleavage Assay. The conversion of [^{3}H]tetradecanoyl-CoA to a hexanes-extractable labeled product was monitored as previously described (*13*). The reaction mixture (100 μL) contained 8 μM tetradecanoyl-CoA (0.1 Ci/mmol) and aldehyde dehydrogenase in 50 mM β-mercaptoethanol and 0.05 M phosphate, pH 7, at 22 °C. Following incubation (2 min), the reaction was terminated by the addition of 5 μL of glacial acetic acid and then extracted with 1.0 mL of hexane containing 5% glacial acetic acid. A total of 0.5 mL of the hexane extract was then counted directly in 10 mL of Cytoscint with a 45% efficiency (92 cpm/pmol of ^{3}H-labeled

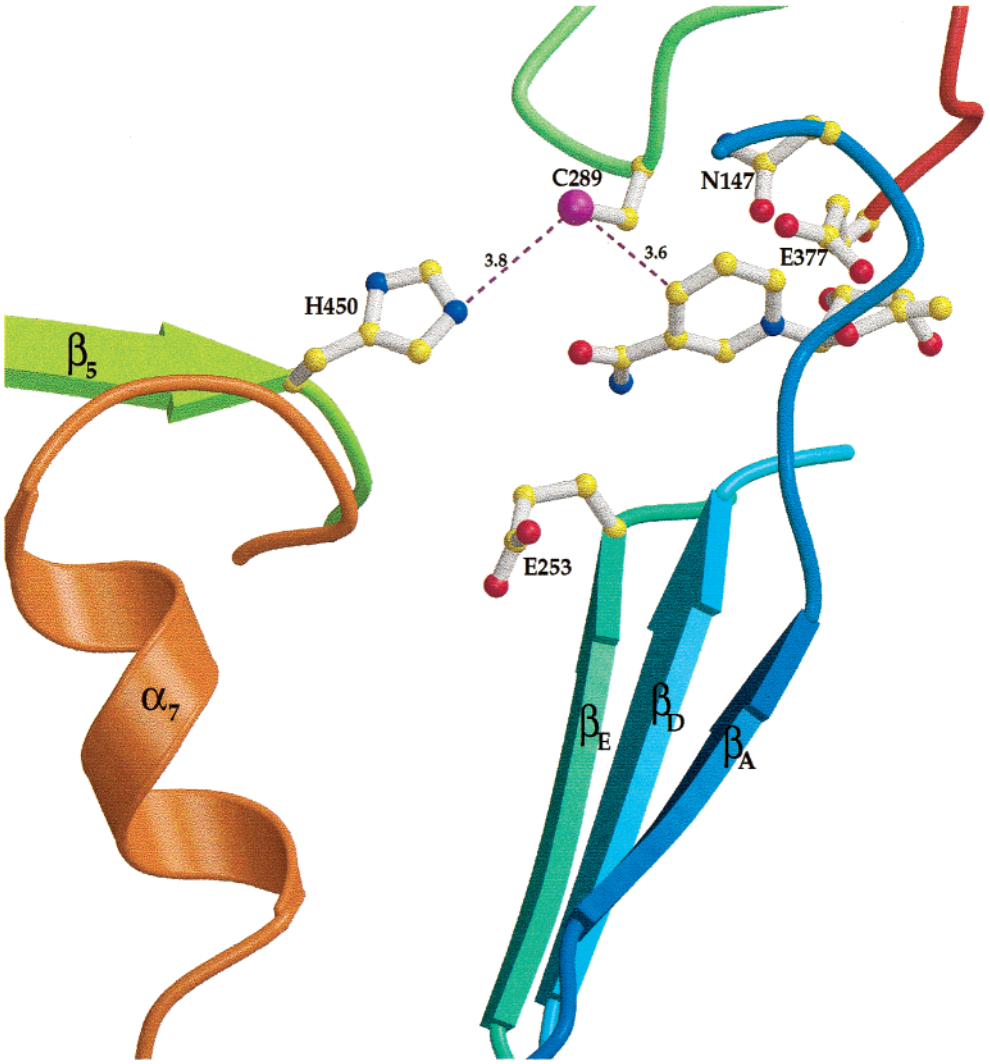

FIGURE 1: Active site of *V. harveyi* ALDH drawn using the programs Molscript (*18*) and Raster 3D (*19*). The polar residues implicated in the catalytic mechanism are shown along with the nicotinamide part of NADP. Hydrogen bond distances are indicated with a dashed line.

product). Radioactivity of the blank (minus enzyme) incubated for the same length of time was subtracted from the sample values. Activity is given in (pmol/min/μg) of enzyme.

Labeling of Polypeptides with [^{3}H]Tetradecanoyl-CoA. The enzyme in 50 mM phosphate (pH 7.0), 0.2 M NaCl, 10% glycerol (v/v), and 0.25 mM dithiothreitol was incubated with 6 μM [^{3}H]tetradecanoyl-CoA (20 Ci/mmol). At different times, aliquots were diluted 1:1 into SDS−sample buffer (2.5% SDS, 25% glycerol, 0.12 M Tris-chloride, pH 6.8, containing 5 mM *N*-ethyl maleimide), and the protein was separated by SDS−PAGE. The gel was stained with before autoradiography. In some instances the bands were excised, dissolved in hyamine hydroxide overnight and then counted in 10 mL of CytoScint. A total of 18 400 cpm corresponds to 1 pmol of ^{3} H-labeled tetradecanoic acid incorporated into the protein.

Chemical Modification. For testing the inactivation of wild-type and mutant ALDHs by *N*-ethylmalemide (NEM), the enzymes, stored in 10 mM β-mercaptoethanol, were initially dialyzed overnight against 30% glycerol, 50 mM phosphate buffer, pH 7, and 10^{-4} M β-mercaptoethanol to lower the level of free thiol groups and the enzyme diluted in the same buffer to 1 μM. The reaction was initiated by adding the enzyme and 2 mM NEM in 0.05 M phosphate, pH 7.0, in a 1:1 ratio. At different times, the reaction was terminated by mixing in a 1:1 ratio 40 mM β-mercaptoethanol, 0.05 M phosphate, pH 7.0. The activity was then determined under standard conditions with saturating concentrations of NAD and dodecanal.

RESULTS

The location of the key polar residues and the nicotinamide moiety of NADP in Vh-ALDH are shown in Figure 1. Despite the weak electron density for the nicotinamide moiety the position of this region of the cofactor is almost identical in Vh-ALDH, ALDH2, and ALDH9 (*3*, *4*, *8*). The distance between the C4 atom of niacin and the Cys nucleophile (C289) is ideal for transfer of a hydride ion from a thiohemiacetal derivative to NADP indicating that the NADP is bound in a functional conformation.

A Histidine Residue in Proximity to the Active Site Nucleophile. Figure 1 also shows that the ϵ-imino group (NE2) of H450 is close enough (3.8 Å) to form a weak hydrogen bond with the Cys nucleophile. This result was unexpected since His residues are not located in proximity to the active site Cys nucleophile in any of the other ALDHs whose crystal structures have been determined (*2*−*7*). Moreover, two Glu residues (E253 and E377 in Vh-ALDH)

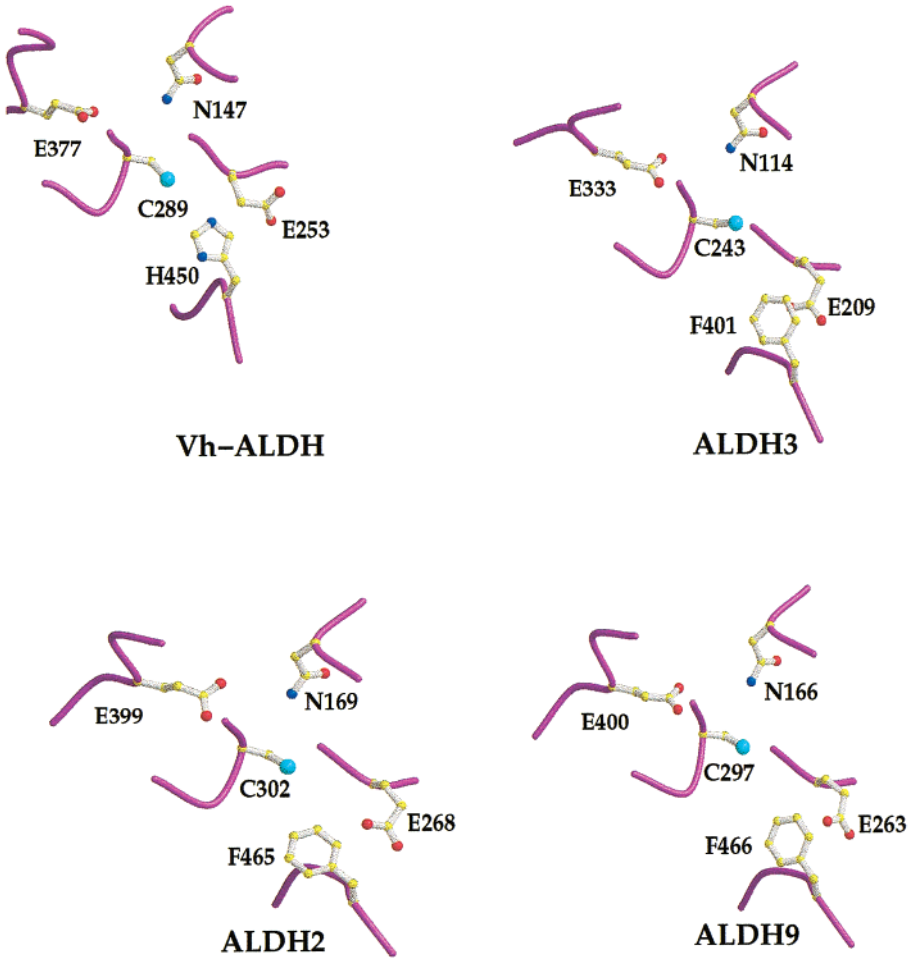

FIGURE 2: Comparison of the relative locations of the key polar residues implicated in the dehydrogenase mechanism in Vh-ALDH, ALDH2, ALDH3, and BALDH (ALDH9). Two Glu, one Asn and one Cys residue are present in the active site of all enzymes, however, only Vh-ALDH has a His residue in the active site while the other ALDHs have a Phe residue at the same relative position. Structural information for the ALDHs was from refs *2*−*4* and *8*.

implicated in increasing the nucleophilicity of the Cys nucleophile in other ALDHs (*10*, *20*) are conserved in the same relative location in the active site of Vh-ALDH and other ALDHs (Figure 2). The Cys nucleophile in Vh-ALDH is in much closer contact to the His residue (3.8 Å) than the Glu residues (6 to 9 Å) and allows the His residue to function directly as a general base with only a minor change in conformation. Figure 2 also shows that a Phe residue and not a His residue is conserved at the same location in ALDH2, ALDH3, and ALDH9. A Phe residue is also present at this location in ALDH1 (*5*) and *S. mutans* ALDH (*6*). Although the same region of retinal ALDH was too flexible to elucidate the structure (*7*), a Phe residue is present in retinal ALDH in the primary sequence at a position corresponding to that of H450 in Vh-ALDH.

To determine if H450 takes part directly in the catalytic mechanism and affects the nucleophilicity of the Cys nucleophile, H450 was mutated to residues (Asn or Gln) with similar structure and polarity and the properties of the ALDH mutants investigated. Asn and Gln mutations were chosen as the oxygen or nitrogen atoms of their amide side chains are isosteric with the ND1 and NE2 nitrogen atoms, respectively, of the imidazole ring of His. Since the NE2 and not the ND1 nitrogen atom of histidine is in proximity (3.8 Å) to the Cys nucleophile, Gln should be able to more readily substitute for His than Asn and thus have a less detrimental effect than Asn on enzyme function if His functions to increase the reactivity of the Cys nucleophile.

Aldehyde Dehydrogenase Steady-State Kinetics. Purification of the H450Q and H450N mutants to homogeneity by a single chromatography step on 2′5′-ADP Sepharose demonstrated that the interaction of the mutants with NADP was still relatively strong. Table 1 gives the kinetic properties with dodecanal at saturating concentrations. The K_m for NAD is relatively unaffected by these mutations remaining within two- fold of that for the wild-type enzyme while the K_m for NADP increased between 6- and 15-fold but still remains relatively low (10−25 μM) explaining the binding to 2′-5′-ADP Sepharose. A decrease in k_{cat} of about 10- and 30−80-fold for H450Q and H450N mutants, respectively, can readily be observed compared to the turnover number of wild-type enzyme with the corresponding nucleotide.

As a low K_m for dodecanal makes it difficult to study the dependence on aldehyde concentration, two-substrate kinetics were conducted with a shorter chain aldehyde, heptanal, and NAD(P) (Table 2). The Michaelis constants for NAD and NADP with heptanal were similar (within 2-fold) to those obtained with dodecanal. The k_{cat} with heptanal dropped about 10-fold for H450Q and 20-fold with H450N with either NAD or NADP as cosubstrate. The only significant difference from the results with dodecanal is that the k_{cat} for H450N with NAD is about 4-fold higher. The Michaelis

Table 1: Kinetic Parameters for WT Vh-ALDH and Mutants with Dodecanal as Substrate[a]

	NAD			NADP		
enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
WT[b]	392 ± 20	3400 ± 89	9 ± 0.5	1.6 ± 0.3	550 ± 3.0	330 ± 64
H450Q	690 ± 126	289 ± 24	0.42 ± 0.08	25 ± 5	58 ± 4.6	2.3 ± 0.4
H450N	224 ± 14	40 ± 0.7	0.18 ± 0.01	9.4 ± 1.7	21 ± 1.1	2.2 ± 0.4

[a] Apparent K_m values and k_{cat} values (± standard errors) were determined in 50 mM sodium potassium phosphate buffer, pH 8.0, at saturating concentrations of dodecanal (0.1 mM) at 25 °C. [b] Data taken from Zhang et al. (*21*).

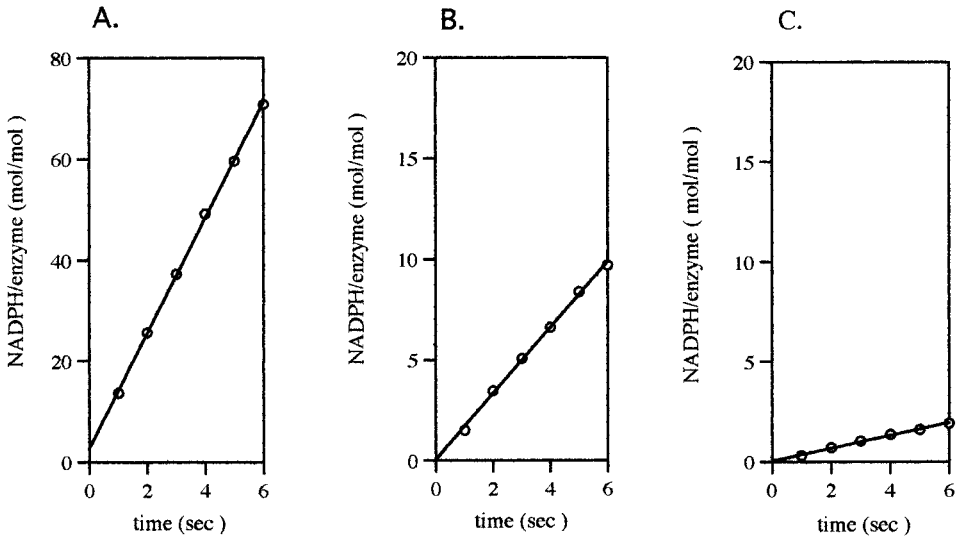

FIGURE 3: Formation of NADPH in the presteady and steady state by Vh-ALDH and the H450Q and H450N mutants. Fluorescence emission at 460 nm of 0.1 μM ALDH (A), 0.5 μM H450Q (B) or 1 μM H450N (C) in 1 mM NADP in 50 mM phosphate buffer, pH 8, was followed with time after injection of 3 μL of 0.01 M dodecanal into 300 μL of assay mixture at zero time. The fluorescence baseline before injection of aldehyde was subtracted from the final fluorescence readings. NADPH production was calculated using a standard curve for fluorescence of NADPH at 460 nm. Fluorescence readings were converted into moles of NADPH and divided by the moles of the dimeric ALDH of 110 kDa. A presteady state burst of 2 mol of NADPH/mol of enzyme can be observed for the wt ALDH (A) while only the steady-state release of NADPH can be observed for either the H450Q (B) or the H450N (C) mutant.

Table 2: Kinetic Constants for Native and Mutant Aldehyde Dehydrogenases[a]

enzyme	K_a (NAD^+) (μM)	K_b (C_7Ald) (μM)	k_{cat} (min^{-1})	k_{cat}/K_a (μM^{-1} min^{-1})	k_{cat}/K_b (μM^{-1} min^{-1})
			NAD^+		
WT[b]	205	190	4800	23	25
H450Q	680	155	400	0.59	2.6
H450N	480	4400	160	0.33	0.036
			$NADP^+$		
WT[b]	1.2	8.6	530	440	62
H450Q	12.7	15.6	60	4.7	3.9
H450N	7.7	770	31	4.1	0.041

[a] Reactions were performed in 50 mM sodium potassium phosphate buffer, pH 8.0, at 25 °C. Michaelis constants for NAD^+, $NADP^+$, and heptanal were determined using two substrate kinetics as described in Materials and Methods. Standard errors for the Michaelis constants and k_{cat} calculated from the secondary plots of slopes and intercepts versus the second substrate were between 5 and 18% of the indicated values. [b] Data taken from Zhang et al. (*21*).

constants for heptanal with NAD and NADP for the H450Q mutant remained remarkably close to those for the wild-type enzyme with the same nucleotide. In contrast, the Michaelis constants for heptanal with the H450N mutant were 30−100-fold higher than those of the wild-type enzyme or the H450Q mutant. These results demonstrate that mutation of H450 to Gln causes a substantial loss of catalytic activity in terms of the turnover rate and that on mutation to Asn the turnover number further decreases along with a weakening of the interaction with aldehyde. A comparison of the rate of the reaction (k_{cat}/K_b) at low aldehyde concentration and high NAD(P) concentration shows that the H450Q mutant has about 10−15-fold lower activity than the wild-type ALDH while the activity of the H450N has dropped about 10^3-fold.

Rate-Limiting Step. A presteady-state burst of 2 mol of NADPH/mol of the dimeric ALDH with dodecanal as substrate has shown that the rate-limiting step for the wild-type enzyme occurs after formation of NADPH (*22*). On the basis of steady-state kinetics, it has been proposed that the rate-limiting step is the release of NADPH (*23*). However, a difference in the rate of reaction with deuterated and nondeuterated acetaldehyde has raised the possibility that hydride transfer may at least be partially rate limiting with short-chain aldehydes (*24*).

Figure 3 shows that a burst of NADPH cannot be observed in the presteady state for either the H450Q or the H450N mutant with dodecanal as substrate, indicating that the rate-limiting step for the mutants now occurs either at or prior to the transfer of the hydride ion to NADP. Comparison of the rates of oxidation of deuterated and nondeuterated acetaldehyde for the H450Q mutant shows that the two substrates are cleaved at similar rates (Table 3). In contrast, the nondeuterated acetaldehyde is oxidized more rapidly than the deuterated acetaldehyde by the wild-type ALDH. These

Table 3: Deuterium Isotope Effect[a]

enzyme	K_m (mM) acetaldehyde	k_{cat} (min^{-1})
WT		
nondeuterated	23	3100
deuterated	23	1300
H450Q		
nondeuterated	11	470
deuterated	14	600

[a] Apparent K_m and k_{cat} were measured in 50 mM sodium potassium phosphate buffer, pH 8.0 at saturating concentration of NADP (1 mM).

Table 4: Esterase and Thioesterase Activities

enzyme	esterase activity[a] (pmol/min/μg)	thioesterase activity[b] (pmol/min/μg)
WT	520	160
WT (+100 μM NADP)	80	3
WT (+100 μM NADPH)	240	8
H450Q	nd[c]	4
H450N	nd	0.1

[a] Rate of cleavage of 50 μM *p*-nitrophenyl acetate at pH 8.0. [b] Rate of cleavage of 5 μM tetradecanoyl-CoA at pH 7.0. [c] Not detected (<3 pmol/min/μg).

results contrast sharply with those observed for the E377Q mutant in which the rate of cleavage of nondeuterated acetaldehyde is four times the rate of cleavage of deuterated acetaldehyde (*24*), indicating that hydride transfer is the rate-limiting step. The results for the H450Q mutant show that the rate-limiting step must occur prior to hydride transfer and formation of NADPH and suggests that the nucleophilic attack by the thiol group of cysteine on the aldehyde is now the slowest step consistent with H450 increasing the nucleophilicity of the Cys nucleophile.

Esterase and Thioesterase Activities of ALDH. The *V. harveyi* ALDH has previously been shown to possess a thioesterase activity with tetradecanoyl-CoA as substrate (*17*, *24*). As esterase activity using *p*-nitrophenylacetate as substrate has routinely been evaluated with other ALDHs (*20*), the H450Q and H450N mutants and wild-type ALDH were also tested for esterase activity. Table 4 shows that neither mutant had detectable esterase activity even though the wild-type enzyme can readily cleave *p*-nitrophenyl acetate. Using tetradecanoyl-CoA as substrate, a low level of activity could be detected with the H450Q mutant with the H450N mutant giving a response very close to background levels even if very high amounts of enzyme were assayed. Clearly mutation of H450 has a major effect on both esterase and thioesterase activities with only 2−3% of the thioesterase and <1% of the esterase activity retained by the H450Q mutant.

As NAD activates the esterase activity of ALDH2 and inhibits the esterase activity of ALDH3 (*11*, *20*), the effects of NADP and NADPH on the esterase and thioesterase activities of Vh-ALDH were also tested (Table 4). Both the reduced and oxidized nucleotide inhibit the esterase activity of the wt ALDH analogous to the effects observed with ALDH3. For the thioesterase activity, NADP inhibited the wt activity while the activity of H450Q (and H450N) was too low to be tested for any effect of added nucleotides.

Detection of an Acylated Enzyme Intermediate. Although a labeled acylated derivative would be expected to be formed

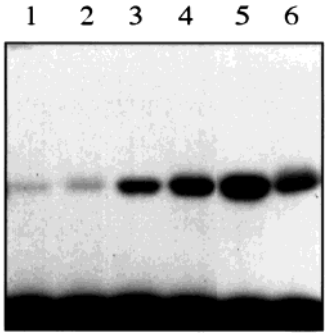

FIGURE 4: Acylation of Vh ALDH and H450 mutants with [^{3}H]-tetradecanoyl-CoA. Enzyme samples (2 μg) were incubated in 25 μL of 0.2 M NaCl, 50 mM phosphate, pH 7, containing 10% glycerol, 0.25 mM dithiothreitol, and 6 μM [^{3}H]tetradecanoyl-CoA for 30 s (lanes 1, 3, 5) and 10 min (lanes 2, 4, 6) before dilution into SDS sample buffer and 80% of the sample analyzed by SDS−PAGE and autoradiography. Lanes 1 and 2, wt ALDH; lanes 3 and 4, H450N; lanes 5 and 6, H450Q. The solvent front containing the free tetradecanoyl-CoA is shown at the bottom as a point of reference.

on reaction of Vh-ALDH with [^{3}H]tetradecanoyl-CoA, direct trapping of this intermediate has proven difficult as this can only be accomplished if significant levels of the acylated intermediate are present in the steady state and quenching of the reaction is more rapid than the rate of deacylation. Such studies have been extensively conducted with the *P. phosphoreum* fatty acid reductase complex which catalyzes the release, activation, and reduction of tetradecanoic acid to the tetradecanal substrate required for the luminescence reaction in light-emitting bacteria (*13*, *25*). These studies have shown that a labeled enzyme with tetradecanoyl groups attached to a serine or cysteine residue intermediate can be detected on SDS−gel electrophoresis after reaction with [^{3}H]-tetradecanoyl-CoA. Our earlier attempts to label the wild-type Vh-ALDH with [^{3}H]tetradecanoyl-CoA resulted in a very low level of incorporation of radioactivity in the polypeptides after SDS−gel electrophoresis (*17*). However, a much higher level of tetradecanoyl groups could be incorporated into either the H450Q or the H450N mutant than the wild-type enzyme both after 30 s and 10 min of incubation with [^{3}H]tetradecanoyl-CoA (Figure 4). As high levels of enzyme must be used in the acylation procedure so as to detect the protein on SDS−gels and only low amounts of tetradecanoyl-CoA can be added due to formation of micelles, the relatively low level of acylation of the wt enzyme may simply be due to cleavage of the tetradecanoyl-CoA before termination of the reaction. Alternatively, the quenching reaction may not be rapid enough to prevent deacylation of a substantial part of the acylated intermediate before the protein is denatured. It is possible that on quenching of the reaction with SDS, the long hydrophobic dodecyl chain of SDS immediately blocks acyl-CoA binding while unfolding of the enzyme requires a longer time during which the enzyme is deacylated.

As the H450Q mutant was more strongly labeled after 30 s than 10 min, and the reverse appeared to be the case for the H450N mutant, the incorporation of labeled tetradecanoyl groups for different time intervals was followed as shown in Figure 5 The maximum level of acylation was reached within 30 s for the H450Q mutant and then slowly decreased from 2 to 10 min of incubation (Figure 5B). In contrast, the H450N mutant takes up to 1.5 min to reach a maximum level

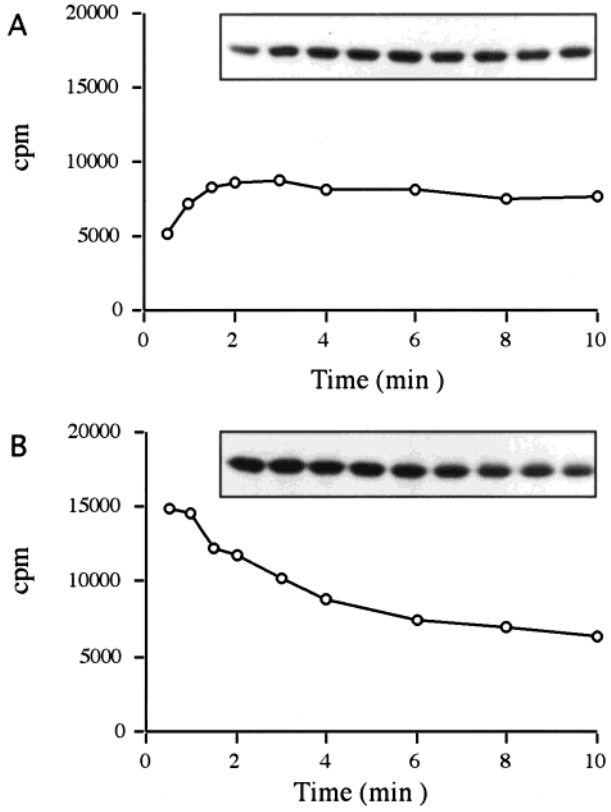

FIGURE 5: Time dependence of acylation of the H450N and H450Q mutants with [^{3}H]tetradecanoyl-CoA. Samples of H450N (A) and H450Q (B) were acylated as described in Figure 4 except the reaction was stopped at the indicated times (30 s to 10 min) before electrophoresis and autoradiography (insets) as described in Materials and Methods. The gel bands were excised, dissolved in 0.4 mL of 90% hyamine hydroxide overnight at 37 °C and then counted in 10 mL of CytoScint. The counts (cpm) reflect the total amount of counts present in the 1.6 μg (14 pmol) of protein applied to the gel where 1.8×10^4 cpm is equal to one pmol of tetradecanoic acid incorporated on the protein.

of acylation and the level of acylation decreased only to a small degree during the later part of the reaction (Figure 5A). As acylation will depend on the nucleophilic attack of the Cys residue at the active site on the carbonyl of tetradecanoyl-CoA, the results indicated that the rate of acylation of the H450Q mutant was more rapid than that of the H450N mutant and suggest that the reactivity of the active site Cys was higher in the H450Q mutant than in the H450N mutant. It should be noted that the apparent level of acylation of both mutants was still quite low. Approximately 0.05 mol of tetradecanoyl residues were incorporated/mol of mutant ALDH under optimal conditions. This low level of acylation was consistent with the rate of deacylation being faster than the rate of acylation.

The effects of NADP as well as NADPH on the level of acylation of the wild-type and mutant ALDHs were also investigated (Figure 6). NADP decreased the level of acylation for both the H450Q and the H450N mutant as well as the wild-type enzyme while NADPH appeared to have a much smaller effect on the relative levels of acylation. The same results were obtained if the mutant and wild-type enzymes were first acylated for l min and then mixed for an additional minute with NADP or NADPH. Although the lower level of acylation could possibly reflect the stimulation of the deacylation step by NADP, it is more likely that NADP

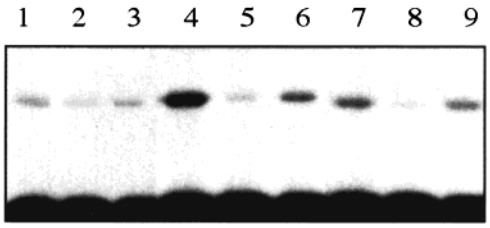

FIGURE 6: Effect of NADP(H) on acylation of Vh-ALDH, H450Q, and H450N mutants. Samples of Vh-ALDH (lanes 1−3), H450Q (lanes 4−6), and H450N (lanes 7−9) were acylated as described in Figure 5 in the presence of 100 μM NADP (lanes 2, 5, 8) and 100 μM NADPH (lanes 3, 6, 9) or in the absence of nucleotides (lanes 1, 4, 7). The reaction was stopped after 1 min by dilution into SDS sample buffer before SDS−PAGE and autoradiography.

Table 5: Tetradecanoyl-CoA Reductase Activity

enzyme	amount assayed (μg)	aldehyde produced (pmol)[a]	specific activity (pmol/min/μg)
Wt	0.02	5.7	280
H450Q	0.1	4.0	40
H450N	1.0	2.7	3

[a] Aldehyde produced in 1 min on incubation of indicated amount of enzyme in 5 μM tetradecanoyl-CoA and 10 μM NADPH measured in a luminescence coupled assay system as described in Materials and Methods. Background response was ~1 pmol of aldehyde in these assays.

blocks acylation as NADP decreases both the esterase and thioesterase activities of the wild-type ALDH (Table 4).

Partial Reversal of the Aldehyde Dehydrogenase Reaction; Tetradecanoyl-CoA Reductase Activity. Vh-ALDH has previously been shown to catalyze the NAD(P)H-dependent reduction of tetradecanoyl-CoA to tetradecanal (*17*). Formation of the aldehyde product is followed by the luminescence response on mixing with bacterial luciferase and $FMNH_2$. Activity can only be detected at very low NADPH concentrations (0.1−10 uM) possibly due to NADPH or NADP blocking the binding of tetradecanoyl-CoA at higher NADPH concentrations. In this assay, maximum activity is observed at low protein concentrations and short times as the fatty aldehyde product can bind to proteins.

Table 5 gives the amount of aldehyde produced after 1 min using the minimal amount of ALDH so that the response is 3−5 times higher than the background response (~0.8 pmol). The calculated specific activities under these specific conditions are in the ratio of 100:17:1 for wt:H450Q:H450N ALDH, respectively. Using a lower level of NADPH (1 μM) gave specific activities for the wt:H450Q:H450N ALDHs of 400:50:7 pmol/min/μg while five times higher amounts of each of the ALDHs gave specific activities of 180:30:1.6 pmol/min/μg, respectively (at 10 μM NADPH), indicating that the relative activities do not change significantly under the different assay conditions. The ratio of the tetradecanoyl-CoA reductase activities for wt:H450Q:H450N ALDH is quite similar to the ratio of the aldehyde dehydrogenase activities (k_{cat}s) with dodecanal of 100:10:4 with NADP and 100:9:1 with NAD given in Table 1.

Reactivity of the Cys Nucleophile with N-Ethylmalemide (NEM). The inactivation of aldehyde dehydrogenase by the thiol-specific reagent *N*-ethylmaleimide arises due to the reaction of the reagent with the active site Cys nucleophile as it is prevented by the addition of the substrates (*17*, *23*). Determination of the rate of inactivation by NEM of the

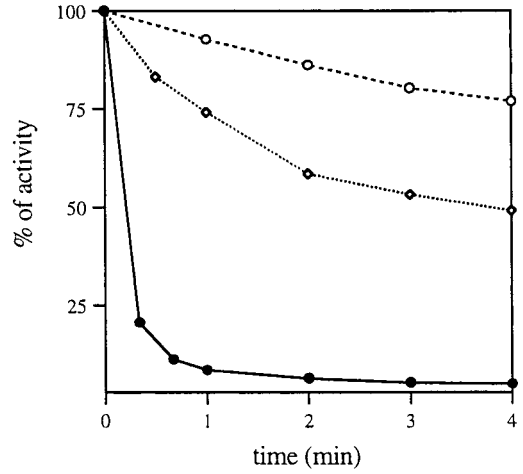

FIGURE 7: Inactivation of Vh-ALDH and H450 mutants with *N*-ethylmaleimide. The enzymes (1 mM) in 50 mM phosphate and 0.1 mM β-mercaptoethanol, pH 7.0, were mixed 1:1 with 2 mM NEM in 50 mM phosphate, pH 7.0, at 25 °C. Aliquots were withdrawn at various times and diluted 1:1 with 40 mM β-mercaptoethanol to stop the reaction and then activity determined in the aldehyde dehydrogenase assay with saturating NAD and dodecanal. Wt (●), H450Q (G), H450N (○).

mutants and wild-type enzyme provides an independent determination of the relative nucleophilicity of the active site Cys residue. Figure 7 shows that the mutants are inactivated at a much slower rate with NEM than the wild-type enzyme. Pseudo-first-order rate constants for inactivation of the mutants with 1 mM NEM can be estimated to be of >5, ~ 0.1, and ~ 0.05 min^{-1} for the wt, H450Q, and H450N ALDHs, respectively, at pH 7. These results indicate that the nucleophilicity of the active site Cys residue is much higher in the wild-type enzyme than in either of the H450 mutants. Moreover, H450N is inactivated at a slower rate than the H450Q mutant consistent with their relative differences in kinetic properties and suggesting that Gln can substitute for His450 better than Asn in terms of maintaining the reactivity of the Cys nucleophile at the active site. This result would support the proposal that Gln can more effectively replace His than Asn as the Gln amide group is isosteric with the ϵ-imino of H450 and assist to a small degree in the deprotonation of the Cys nucleophile.

DISCUSSION

The presence of a His residue in the active site of Vh-ALDH was quite unexpected. A total of 145 proteins are listed as part of an extended family of ALDHs (*1*) with the crystal structures of seven ALDHs having now been determined. Only Vh-ALDH has a His residue located in the active site in proximity to the Cys nucleophile while the other crystalline ALDHs have a Phe residue at the same location.

The identity of the general base increasing the nucleophilicity of the Cys residue forming the thiohemiacetal derivative in the aldehyde dehydrogenase mechanism has been a central question over the past decade. Studies have focused primarily on two highly conserved Glu residues (Glu1 and Glu2) located at the same relative position in the active site of all ALDHs whose structure has been determined including Vh-ALDH. The function of these two specific Glu residues(Glu1, E253/268/209, and Glu2, E377/399/333 in Vh-ALDH/ALDH2/ALDH3, respectively) have been extensively investigated. In Vh-ALDH (*24*) and ALDH2 (*9*, *11*, *20*), the Glu1 residue has been proposed to activate the Cys nucleophile as mutation of the specific Glu residue in either enzyme resulted in a large decrease in esterase activity as well as k_{cat} in the dehydrogenase assay without affecting substrate binding. However, mutation of Glu2(E333) and not Glu1(E209) in human stomach ALDH3 resulted in kinetic properties very similar to those observed for mutants of Glu1 in VH-ALDH and ALDH2 (*20*). A mechanism involving E333 as the general base has also been proposed based on the structure of rat ALDH3 (*10*). Despite the different locations of Glu1 and Glu2 in the active sites of ALDHs in the mechanism, differences in the conformation of bound NAD in the active site in ALDH3 and ALDH2 could readily account for these results (*2*, *3*, *20*).

Recently, a detailed study on the reactivity of the Cys nucleophile (C302) in the glycerol 3-phosphate NADP-specific *S. mutans* ALDH has shown that mutation of Glu1 (E268) did not affect the inactivation of the enzyme with iodoacetamide (*26*). These results also showed that addition of NADP caused a conformational change leading to an increased reactivity of the Cys nucleophile and that E268 was required for this change in conformation with its major role being to activate a water molecule rather than stabilize the Cys nucleophile.

Interestingly, in all cases the Glu residue is located at least 5 Å away from the catalytic thiol group in the mechanism. For ALDH2 and ALDH3, Glu1 and Glu2 are between 6 and 8 Å from the Cys nucleophile. Similar distances are found between the Cys nucleophile and the Glu1 or Glu2 residue in other crystalline ALDHs except for Vh-ALDH. For Vh-ALDH, both Glu1 and Glu2 are located slightly further (7.3−8.4 Å) from the Cys nucleophile than the equivalent Glu residue in the other ALDHs. The Glu1 (E253) residue in Vh-ALDH, in particular, is located the furthest from the Cys nucleophile of any active site Glu residue with its carboxylate oxygen atoms pointed away from the sulfur group of C289. Coupled with the proximity (3.8 Å) of H450 to C289 in Vh-ALDH, these observations raise the question of what residue was responsible for increasing the reactivity of the Cys nucleophile in the catalytic mechanism.

The experiments presented in this paper have provided direct evidence that H450 contributes strongly to the nucleophilicity of C289 in the active site. Analysis of the kinetic properties of two mutants, H450Q and H450N, of Vh-ALDH, demonstrated that the k_{cat} for the aldehyde dehydrogenase activity was decreased about 10- and 30-fold for the H450Q and H450N mutants, respectively. This loss of activity was accompanied by a shift in the rate-limiting step to a step prior to hydride transfer consistent with this step being at least partially controlled by the nucleophilic attack of the active site Cys residue on the aldehyde to form the thiohemiacetal derivative. In addition, large decreases in the tetradecanoyl-CoA reductase, tetradecanoyl-CoA thioesterase, and *p*-nitrophenylacetate esterase activities were observed on mutating the H450 residue with the reductase activity dropping 7- and 90-fold and the thioesterase activity dropping 40- and >1500-fold, for the H450Q and H450N mutants, respectively, while esterase activity could not be detected ($<2\%$ of wt) for either mutant. These results together indicated that the nucleophilicity of the Cys residue (C289) at the active site in the mutants was decreased. Moreover,

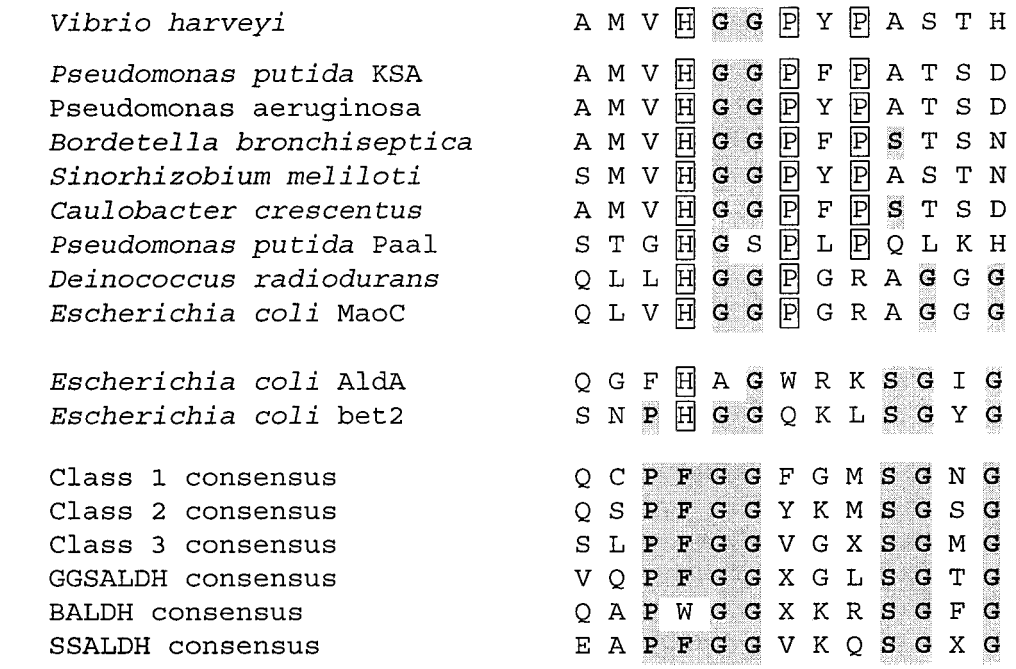

FIGURE 8: Comparison of the sequences of potential ALDHs with a histidine residue in the active site to the consensus sequence found in different families of ALDHs. The residues (PFGG−SG-G) highly conserved between the different ALDH families (*1*) are shaded in gray for the consensus sequences of six of the ALDH families (bottom) and in proteins (top, middle) that contain a His residue at the same sequence position as *V. harveyi* ALDH. Proteins containing the His residue were detected by a Blast search of the protein sequence data bank using the carboxyl terminal half of *V. harveyi* ALDH (top) as well as by scanning the list of ALDHs at the web site of Perozich et al. (*1*) (middle). Four different sequences from *Ps. putida* and three each from *Ps. aeruginosa* and *E. coli* were detected, however, only those with significant differences in sequence are listed. Prelimary sequence data for *C. crescentus*, *Ps. aeruginosa*, *B. bronchoseptica*, and *S. meliloti* were obtained from the Institute for Genomic Research website at http://www.tigr.org. The sequences for *Ps. putida* KSA is only listed as a direct deposit in the gene bank by R. P. Burlingame, A. Maraga, A. Ally, D. Ally., and K. C. Beckman (1995) as are the sequences of *E. coli* MAOC and bet2 by H. Mori (1996) (*1*). Original sources for other sequences were *V. harveyi* (*22*), *Ps. putida* Paal (*28*), *D. radiodurans* (*29*), and *E. coli* AldA (*30*). Consensus sequences for class1 (ALDH1), class 2(ALDH2), class 3(ALDH3), GGALDH (γ-glutamyl semialdehyde dehydrogenase, SSALDH (succinic semialdehyde dehydrogenase), and BALDH (betaine ALDHs, ALDH9) families are taken from Perozich et al. (*1*). Positions in the consensus sequence which are variable within a family are denoted by X. The highly conserved His and Pro residues in the potential ALDHs with histidine in the active site are boxed.

the relative degree of inactivation was less for the H450Q than the H450N mutant consistent with the carbonyl group of Gln being long enough to partially replace the ϵ-imino group of H450 which is in proximity to the thiol group of C289 and thus assist in increasing its nucleophilicity.

Direct evidence for the change in reactivity of the Cys nucleophile was provided by the loss of the ability to inactivate Vh-ALDH with NEM at pH 7. This reagent is specific for thiol groups at pH 7 (*27*) with inactivation due to the reaction of NEM with the Cys nucleophile in the active site (C289) as substrates protect the enzyme from reaction (*23*). Inactivation with NEM of the wt ALDH occurred at a rate at least 20-fold faster than the inactivation of the H450Q mutant which in turn was inactivated with NEM at a rate two- to 3-fold faster than the H450N mutant. The relative rates of inactivation with NEM closely parallel the relative losses in the different functions showing that the H450 residue functions as a general base in the mechanism and helps assist in the (de)protonation of the Cys nucleophile. This result can be contrasted with the experiments on *S. mutans* ALDH in which modification of the Glu1 (E268) residue had little effect on the reactivity of the Cys nucleophile (*26*).

Elucidation of why a His residue is important to activate the Cys nucleophile as part of the mechanism in Vh-ALDH but is not part of the enzyme mechanism of all other nonphosphorylating ALDHs that have been studied is a question of major interest. Only one other protein in the extensive list of ALDHs compiled by Perozich et al. (*1*) is closely related in sequence to Vh-ALDH. Interestingly this enzyme, *Pseudomonas putida* ketosemialdehyde dehydrogenase (KSA-ALDH) also contains a His residue in the same primary sequence site as H450 of Vh-ALDH. The close sequence relationship between Vh-ALDH and KSA-ALDH (~50% sequence identity) and the presence of a His residue in the active site may suggest that these two ALDHs have similar substrate specificities. In contrast, comparison of their sequences to other ALDHs in the list of 145 ALDHs compiled by Perozich et al. (*1*) shows that Vh-ALDH has less than 25% sequence identity with any other ALDH and that the vast majority have a Phe residue at this site.

A scan of all the protein sequence data bank including the unpublished genomic sequences for related sequences using the carboxyl terminal half of Vh-ALDH as well as a screening of the list of ALDHs compiled by Perozich et al. (*1*) for a His residue at the same primary sequence location as H450 did, however, demonstrate that a few proteins have a His residue at this site. Figure 8 gives a list of the genomic sources coding for ALDH-related proteins with a His residue at the same site as H450 of Vh-ALDH that could be recognized by this screening procedure. All of the related sequences derive from bacterial sources with much of the information originating from the unpublished data of the Institute for Genomic Research. Comparison of the sequences

flanking the histidine residue to that for the consensus sequence found in six major classes of ALDHs shows some distinct differences. In particular, this region is highly conserved and generally contains a motif of PFGGxxxSGxG in most ALDHs. In contrast, the enzymes with a His residue in place of the Phe residue generally only retain the two adjacent Gly residues of this motif and often have the sequence motif HGGPxP. Two of the enzymes from *E. coli* (aldA and bet2) were unrelated in sequence to Vh-ALDH and were selected by screening the list of ALDHs compiled by Perozich et al. (*1*) for a His at the equivalent position as H450 in Vh-ALDH and not by sequence homologies. Interestingly, both enzymes contain the downstream SGxG part of the standard motif and thus appear to have a sequence more closely related to the PFGGxxxSGxG consensus sequences for ALDHs. In contrast, a number of the species contain proteins that are much more closely related to Vh-ALDH with sequence identity greater than 40% with Vh-ALDH. Indeed, six species including *V. harveyi* ALDH contain the sequence (A/S)MVHGGP(Y/F)P(A/S)(S/T) raising the possibility that all enzymes with this sequence motif have the same or similar substrate specificity. Unfortunately, except for Vh-ALDH, functional information about these proteins is extremely limited. Although the protein from *Ps. putida* (KSA) has been designated as a ketoglutarate semialdehyde dehydrogenase, only the sequence has been deposited in the database. Further studies on the enzymic properties in vitro and the functional role in vivo of the ALDHs with a His residue as part of the catalytic mechanism will be important to understand why a histidine residue has evolved as a general base to increase the reactivity of the Cys nucleophile in such a limited number of ALDHs.

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