Involvement of Cysteine 289 in the Catalytic Activity of an NADP$^+$-Specific Fatty Aldehyde Dehydrogenase from *Vibrio harveyi*[†]

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ABSTRACT: Fatty aldehyde dehydrogenase (Vh.ALDH) from the luminescent bacterium, *Vibrio harveyi*, may be implicated in controlling luminescence as it catalyzes the oxidation of the fatty aldehyde substrate for the light-emitting reaction. On the basis of the amino-terminal sequence of Vh.ALDH, a degenerate probe was used to screen a genomic library of *V harveyi* in pBR322, a positive clone was selected containing the Vh.ALDH gene and expressed in *Escherichia coli*, and the enzyme was purified to homogeneity. Although the sequence of the *V. harveyi* ALDH significantly diverged from other aldehyde dehydrogenases, mutation of a conserved cysteine implicated in catalysis completely inactivated the enzyme without loss of its ability to bind nucleotides, consistent with a catalytic role for this residue. Using absorption and fluorescence assays for NAD(P)H, it was shown that NAD$^+$ and NADP$^+$ bound to the same site and that saturation of Vh.ALDH with NADP$^+$ occurred with a Michaelis constant (K_m = 1.4 μM) over 40 times lower than that reported for other aldehyde dehydrogenases. Although *V. harveyi* aldehyde dehydrogenase is unique in terms of its high specificity for NADP$^+$, the identification of a catalytic conserved cysteine in Vh.ALDH clearly indicates that a highly related mechanism and structure have been retained among even the most diverged aldehyde dehydrogenases.

Fatty aldehyde dehydrogenase (Vh.ALDH) from the bioluminescent bacterium, *Vibrio harveyi*, a homodimer of 110 kDa, catalyzes the oxidation of long-chain fatty aldehyde, a substrate for the luminescent reaction (Bognar & Meighen, 1978). The enzyme has also been demonstrated to have a very low level of acyl-CoA reductase activity (Byers & Meighen, 1984), thus possibly implicating it in the synthesis as well as the removal of fatty aldehydes, even though a fatty acid reductase complex that synthesizes fatty aldehyde is also present in luminescent bacteria (Riendeau et al., 1982; Rodriguez et al., 1983a,b; Miyamoto et al., 1988). Originally identified as an NAD$^+$-specific aldehyde dehydrogenase with a low activity with NADP$^+$ (Meighen et al., 1976), it was subsequently demonstrated to bind tightly to 2′,5′-ADP Sepharose and shown to require lower concentrations of NADPH than NADH for its reductase activity (Byers & Meighen, 1984), indicating that its preferred cofactor for dehydrogenase activity may actually be NADP$^+$. This result is very interesting in that most aldehyde dehydrogenases from both prokaryotic and eukaryotic organisms are NAD$^+$-specific with one group of aldehyde dehydrogenases (with few presently documented examples) having a preference for NADP$^+$ (Lindahl & Evces, 1984; Lindahl et al., 1985; Aurich et al., 1987; Evces & Lindahl, 1989; Heim & Strehler, 1991; Sreerama & Sladek, 1993). Moreover, in all the latter cases, the interaction of NADP$^+$ with the enzyme was relatively weak with the lowest reported K_m for NADP$^+$ being greater than 60 μM (Aurich et al., 1987; Lindahl, 1992).

Amino acid sequence alignment of 16 aldehyde dehydrogenases from different species shows that 23 amino acids are invariant (Hempel et al., 1993). Among the invariant residues, there is only one conserved cysteine residue which corresponds to cysteine 302 from class 1 and 2 mammalian aldehyde dehydrogenases.[1] This residue was first identified in the human cytoplasmic aldehyde dehydrogenase (Hempel & Pietruszko, 1981; Hempel et al., 1982) using iodoacetamide and was shown to react with NAD$^+$ (von Bahr-Lindström et al., 1985) and aldehyde analogs (Pietruszko et al., 1991). This cysteine residue is generally considered as the best candidate to form a thiohemiacetal covalent intermediate with aldehyde in the active site of aldehyde dehydrogenases (Hempel et al., 1993; Wang & Weiner, 1995; Farrés et al., 1995). Site-directed mutagenesis analysis also verified this cysteine residue as an active site nucleophile in mammalian aldehyde dehydrogenase (Farres et al., 1995).

In this study, the *V. harveyi* aldehyde dehydrogenase gene was cloned, sequenced and expressed at high levels in *Escherichia coli*, and the properties and active site of the purified recombinant aldehyde dehydrogenase were analyzed. The Vh.ALDH gene encodes a 54.5 kDa polypeptide, whose sequence diverged significantly from other aldehyde dehydrogenases (Weretilnyk & Hanson, 1990; Norlund & Shingler, 1990; Heim & Strehler, 1991; Cook et al., 1991) but still retained a conserved cysteine residue (Hempel et al., 1993) required for activity but not coenzyme binding. Kinetic studies demonstrated that the K_m for NADP$^+$ was remarkably low, showing that the nucleotide specificity of *V. harveyi* aldehyde dehydrogenase is unique.

MATERIAL AND METHODS

Materials. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Labs or Pharmacia. [^{35}S]-

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[1] The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number U39638.

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Methionine (800 Ci/mmol) and [^{35}S]dATP (1400 Ci/mmol) were obtained from Du Pont-New England Nuclear. $NADP^+$ and NAD^+ were purchased from Sigma Chemical Co. Aldehydes were from Aldrich. Dialysis tubes was purchased from Spectrum Medical Industries Inc. The bacterial strains used in these studies were *E. coli* K38 and *V. harveyi* B392.

DNA Sequencing. The nucleotide sequence of Vh.ALDH was obtained using the dideoxy chain termination method (Sanger et al., 1977) after transfer of the DNA into M13. Both strands of DNA were sequenced using synthetic primers (Sheldon Biotechnology Centre, McGill University) located at positions approximately 300 bases apart.

Expression of Vh.ALDH using the Bacteriophage T7 RNA Polymerase/Promoter System. E. coli K38 cells were transformed with the pT7-3 plasmid containing recombinant DNA and the pGP1-2 plasmid coding for T7 RNA polymerase under control of a temperature sensitive repressor (Tabor & Richrdson, 1985). For [^{35}S]methionine labeling, the cells were grown in LB media with 40 μg of ampicillin and kanamycin/mL at 30 °C up to A_{590} = 0.5 and were centrifuged. The pellet was washed with M9 minimal medium and centrifuged. Cells were suspended in M9 medium supplemented with 20 μg of thiamine/mL and 0.01% of 19 amino acids (minus methionine) and then grown for 20 min. Expression of T7 RNA polymerase was induced by heating to 42 °C. Rifampicin was then added to a final concentration of 200 μg/mL to block *E. coli* RNA polymerase followed by labeling of protein with [^{35}S]methionine. The samples were then resolved by SDS−PAGE.

For purification of Vh.ALDH, cells were grown in enriched media (2% tryptone/1% yeast extract/0.5% NaCl/0.2% glycerol/50 mM KPO_4, pH = 7.2, and 50 μg of ampicillin and kanamycin/mL) at 27 °C up to A_{590} = 1.5, the temperature was shifted to 42 °C, and rifampicin was added. The cells were then grown for 1 h at 27 °C, centrifuged, resuspended in 50 mM phosphate buffer and 10 mM β-mercaptoethanol, pH 7, at 4 °C, and lysed by sonication. After centrifugation, the supernatant was used for purification.

Enzyme Purification. Recombinant Vh.ALDH was purified by a two-step procedure involving Cellex D ion exchange chromatography and 2′,5′-ADP Sepharose affinity chromatography as reported previously for the native enzyme (Byers & Meighen, 1984). For small samples it was possible to apply the extract directly to a 2′,5′-ADP Sepharose and elute homogenous enzyme as determined by SDS−PAGE.

Protein Concentration. Protein concentration was determined with the Bio-Rad protein determination kit, using bovine serum albumin as a standard.

Spectrophotometric Assays. Vh.ALDH activity was routinely measured at 24 °C (±1 °C) from the initial increase in absorbance with time at 340 nm after addition of an aliquot of enzyme to 1.0 mL of 50 mM phosphate buffer, pH 8, containing 0.002% (v/v) dodecanal (0.1 mM) and 1.5 mM NAD^+. Aldehyde stock solutions (0.2%) were prepared in 2-propanol. The activity of Vh.ALDH at other pHs was measured in either 50 mM potassium phosphate (pH 6.2−8) or 50 mM sodium pyrophosphate (pH 8−9.5) buffer.

Fluorescence Assays. Measurement of Vh.ALDH with low concentrations of $NADP^+$ were performed on a Hitachi F-3010 fluorimeter with 0.002% dodecanal in 50 mM phosphate, pH 8, at 25 °C. Production of NADPH was monitored by measuring the emission at 460 nm after excitation at 340 nm. The wavelength dispersion was 5 nm for both excitation and emission.

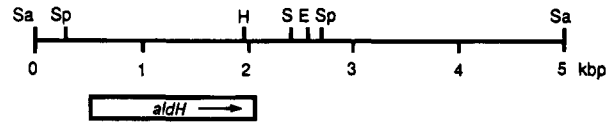

FIGURE 1: Restriction map of cloned *V. harveyi* DNA containing the ALDH gene (*aldH*). Restriction sites are labeled as follows: Sa, *Sal*I; Sp, *Sph*I; H, *Hin*dIII; S, *Sac*I; E, *Eco*RI.

Site-Directed Mutagenesis. An *Sph*I fragment in M13 containing the Vh.ALDH gene was mutated on the basis of the phosphothiorate method (Taylor et al., 1985; Nakamaye et al., 1986) using the Sculptor mutagenesis kit from Amersham International. The codon for cysteine at position 289 (TGT) was altered to TCT (serine). The mutated DNA was transferred to the pT7-3 vector, and the sequence was reconfirmed and expressed as described for native Vh.ALDH.

To test its binding ability, the C289S mutant ALDH was labeled by incubating 14 mL of *E. coli* cells transformed with the pT7-3 vector containing the mutated DNA and the pGP1-2 vector with 300 μCi of [^{35}S]methionine as described above and then lysed by sonication. Free [^{35}S]methionine was removed by repeated dilution and centrifugation through membranes (Amicon) with an exclusion of 30 kDa. The lysate (28.2 μCi) was applied on 2′,5′-ADP Sepharose and washed with five times the column volume of 50 mM phosphate buffer, pH 7, 10 mM β-mercaptoethanol to remove unbound proteins. The column was eluted with 100 μM of $NADP^+$ in the same buffer. Fractions (0.8 mL) were collected, and radioactivity was measured for 10 μL aliquots in 10 mL of CytoScint (ICN) using a Beckman LS 3801 counter.

RESULTS

Cloning and Sequencing of the Aldehyde Dehydrogenase Gene. A genomic library containing the aldehyde dehydrogenase gene was created by restriction of *V. harveyi* DNA with *Sal*I and insertion into the *Sal*I site of pBR322. In order to generate a probe specific for aldehyde dehydrogenase, the enzyme was purified from *V. harveyi* and the amino-terminal sequence was determined. A degenerate probe (23-mer) complementary to the coding region of the N-terminal sequence was used to screen *E. coli* transformed with pBR322 containing a *V. harveyi* genomic library restricted with *Sal*I. Figure 1 shows the map of a 5 kbp *Sal*I restriction fragment inserted in pBR322 isolated from a colony detected with this probe. On the basis of hybridization data with the degenerate probe, the 2.4 kbp *Sph*I fragment was excised and transferred into M13. Determination of the sequence in both directions resulted in the aldehyde dehydrogenase gene being specifically located (Figure 1).

The nucleotide sequence and the predicted amino acid sequence are given in Figure 2. The translated sequence at the amino terminal is in agreement with the 22-residue N-terminal sequence determined from amino acid sequencing except for an alanine residue at position 14 instead of a residue tentatively identified as glutamic acid in the amino acid sequence. A Shine−Dalgarno sequence can readily be recognized upstream of the initiation codon. The open reading frame extends downstream for 1530 nucleotides and codes for a polypeptide of 510 amino acids with a molecular mass of 54.5 kDa. A classical ρ-independent transcription

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                                                         -50  ACCCATACGCGTATGGATTTGAAGTGAAATAAGGACAGTAAGGAATCACA     -1

ATG AAC CCG CAA ACC GAC AAC GTT TTT TAC GCT ACT AAC GCC TTT ACC GGT GAG GCG CTC CCT TTA GCT TTT CCT GTT CAT ACG GAA GTT GAG GTC     96
 M   N   P   Q   T   D   N   V   F   Y   A   T   N   A   F   T   G   E   A   L   P   L   A   F   P   V   H   T   E   V   E   V      32

AAT CAA GCC GCA ACT GCT GCT GCA AAA GTC GCA CGC GAT TTT CGC CGC TTA AAT AAC AGT AAG CGC GCT AGT CTG CTT CGC ACG ATT GCC AGT GAA    192
 N   Q   A   A   T   A   A   A   K   V   A   R   D   F   R   R   L   N   N   S   K   R   A   S   L   L   R   T   I   A   S   E      64

TTA GAG GCA AGA AGT GAT GAC ATT ATC GCC CGA GCC CAT TTA GAA ACG GCG CTT CCC GAA GTA CGC CTG ACA GGA GAA ATC GCT CGT ACA GCG AAT    288
 L   E   A   R   S   D   D   I   I   A   R   A   H   L   E   T   A   L   P   E   V   R   L   T   G   E   I   A   R   T   A   N      96

CAA CTT CGC TTA TTC GCT GAC GTA GTC AAT TCT GGC AGT TAT CAC CAA GCC ATT CTT GAT ACG CCA AAC CCA ACT CGC GCC CCA CTA CCT AAG CCA    384
 Q   L   R   L   F   A   D   V   V   N   S   G   S   Y   H   Q   A   I   L   D   T   P   N   P   T   R   A   P   L   P   K   P     128

GAT ATT CGT CGC CAA CAA ATT GCG CTG GGT CCT GTC GCG GTA TTT GGA GCA TCC AAC TTT CCG TTG GCT TTC TCT GCT GCT GGT GGA GAT ACT GCT    480
 D   I   R   R   Q   Q   I   A   L   G   P   V   A   V   F   G   A   S   N   F   P   L   A   F   S   A   A   G   G   D   T   A     160

TCT GCC CTC GCA GCG GGT TGC CCG GTG ATT GTA AAA GGT CAT ACT GCG CAC CCA GGT ACA AGC CAA ATC GTT GCA GAG TGT ATC GAG CAA GCG CTG    576
 S   A   L   A   A   G   C   P   V   I   V   K   G   H   T   A   H   P   G   T   S   Q   I   V   A   E   C   I   E   Q   A   L     192

AAA CAA GAG CAA CTA CCA CAA GCG ATT TTC ACC TTG CTG CAA GGA AAT CAG CGT GCT TTA GGA CAA GCT CTA GTC AGC CAT CCT GAA ATA AAA GCG    672
 K   Q   E   Q   L   P   Q   A   I   F   T   L   L   Q   G   N   Q   R   A   L   G   Q   A   L   V   S   H   P   E   I   K   A     224

GTG GGC TTT ACT GGA TCG GTT GGT GGT GGA CGC GCC CTG TTC AAC CTA GCG CAC GAG CGA CCT GAG CCA ATC CCG TTC TAT GGT GAA CTT GGT GCG    768
 V   G   F   T   G   S   V   G   G   G   R   A   L   F   N   L   A   H   E   R   P   E   P   I   P   F   Y   G   E   L   G   A     256

ATT AAC CCG ACG TTT ATT TTC CCT TCT GCA ATG AGA GCC AAA GCA GAT TTA GCG GAT CAA TTT GTT GCC TCA ATG ACC ATG GGA TGT GGG CAA TTT    864
 I   N   P   T   F   I   F   P   S   A   M   R   A   K   A   D   L   A   D   Q   F   V   A   S   M   T   M   G   C   G   Q   F     288

TGT ACA AAA CCT GGC GTA GTG TTC GCG CTT AAC ACA CCG GAA ACA CAA GCG TTT ATT GAA ACC GCG CAA TCC CTC ATT CGC CAA CAA TCC CCT TCT    960
 C   T   K   P   G   V   V   F   A   L   N   T   P   E   T   Q   A   F   I   E   T   A   Q   S   L   I   R   Q   Q   S   P   S     320

ACT CTA CTT ACT CCC GGA ATC CGT GAT AGT TAC CAA TCA CAG GTC GTC AGC CGA GGT TCT GAC GAC GGA ATT GAC GTC ACT TTT TCT CAA GCG GAG   1056
 T   L   L   T   P   G   I   R   D   S   Y   Q   S   Q   V   V   S   R   G   S   D   D   G   I   D   V   T   F   S   Q   A   E     352

TCC CCT TGT GTT GCC TCG GCT CTC TTT GTT ACA AGC AGT GAA AAT TGG CGC AAA CAT CCT GCG TGG GAA GAA GAG ATT TTT GGT CCA CAA TCG TTG   1152
 S   P   C   V   A   S   A   L   F   V   T   S   S   E   N   W   R   K   H   P   A   W   E   E   E   I   F   G   P   Q   S   L     384

ATC GTC GTT TGT GAA AAT GTG GCT GAT ATG CTT TCG CTC AGT GAA ATG CTA GCA GGA TCG CTA ACC GCG ACA ATT CAT GCA ACG GAA GAA GAT TAC   1248
 I   V   V   C   E   N   V   A   D   M   L   S   L   S   E   M   L   A   G   S   L   T   A   T   I   H   A   T   E   E   D   Y     416

CCA CAA GTA TCG CAG CTC ATC CCT CGT TTG GAA GAG ATT GCC GGA CGA CTG GTA TTT AAT GGT TGG CCG ACA GGC GTA GAA GTT GGT TAT GCC ATG   1344
 P   Q   V   S   Q   L   I   P   R   L   E   E   I   A   G   R   L   V   F   N   G   W   P   T   G   V   E   V   G   Y   A   M     448

GTG CAT GGC GGT CCA TAT CCC GCA TCA ACC CAT TCG GCT TCG ACT TCT GTT GGT GCC GAA GCC ATT CAT CGT TGG CTA CGT CCG GTG GCT TAT CAA   1440
 V   H   G   G   P   Y   P   A   S   T   H   S   A   S   T   S   V   G   A   E   A   I   H   R   W   L   R   P   V   A   Y   Q     480

GCG TTA CCA GAA AGC TTA CTG CCC GAT TCG TTA AAA GCA GAA AAT CCA TTG GAG ATC GCG CGC GCA GTA GAT GGC AAA GCA GCT CAC AGT TAA GTC   1536
 A   L   P   E   S   L   L   P   D   S   L   K   A   E   N   P   L   E   I   A   R   A   V   D   G   K   A   A   H   S   *            510
                                                                                                                                   1599
ATTGGCTCAAAAAGGCACCAATCGGTGCCTTTTATTTCACGCCACATCTCAAAACCTTCGCTT
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FIGURE 2: Nucleotide sequence and corresponding translated amino acid sequence of the ALDH gene from *V. harveyi*. Nucleotides and amino acids are numbered from the initiation codon. The Shine–Delgarno sequence upstream and a palindromic GC-rich region followed by a poly T region downstream of the ALDH gene are underlined.

Table 1: Purification of Recombinant Aldehyde Dehydrogenase

purification step	volume (mL)	tot. protein (mg)	tot. activity (units)	specific activity (units/mg)
lysate supernatant	700	1190	1194	1
DEAE-cellulose chromatography	150	97.5	1080	11.1
2′,5′-ADP Sepharose chromatography	12.5	18.1	603	33.2

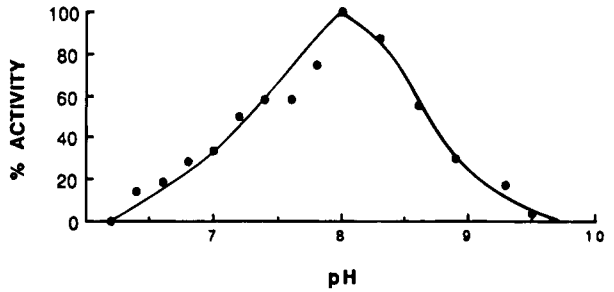

FIGURE 3: Vh.ALDH activity as a function of pH. Activities were measured with NAD^+ using the spectrophotometric assay described in Materials and Methods. Buffers were composed of 50 mM potassium phosphate (pH 6.2−8) or 50 mM sodium pyrophosphate (pH 8−9.5).

termination site consisting of a GC-rich palindrome immediately followed by a poly-T region can easily be recognized about 20 bp downstream of the termination codon.

Expression and Purification of Aldehyde Dehydrogenase. A restriction fragment encompassing the aldehyde dehydrogenase gene extending from the downstream *Sac*I site (at 2.4 kbp, Figure 1) through the upstream *Sph*I site (at 0.3 bp, Figure 1) to a *Sac*I site in the polylinker was excised from M13 containing the *Sph*I restriction fragment and ligated into the *Sac*I site of pT7-3. Two plasmids with the aldehyde dehydrogenase aligned in the forward and reverse directions with respect to the T7 RNA polymerase promoter were isolated with 54 kDa polypeptide being specifically synthesized when the T7 promoter was upstream of the aldehyde dehydrogenase gene (as depicted in Figure 1).

The expression of the aldehyde dehydrogenase in *E. coli* was high enough to allow purification of almost 20 mg of enzyme from 6 L of culture in a two-step purification procedure. A single protein band of 54 kDa was obtained for Vh.ALDH on SDS gel electrophoresis after affinity chromatography on 2′,5′-ADP Sepharose. The specific activity of the purified recombinant aldehyde dehydrogenase (Table 1) is twice as high as that reported previously for the enzyme isolated from the native strain (Bognar & Meighen, 1978); however, this increase reflects the change in assay conditions from pH 7 to pH 8. Figure 3 shows that the activity of aldehyde dehydrogenase increases as the pH is increased, reaching an optimum at pH 8.

Kinetic Properties. Although the apparent Michaelis constants for NAD^+ in the range 100−400 μM have been determined for *V. harveyi* aldehyde dehydrogenase, such studies have not been accomplished with $NADP^+$ due to the limits in the sensitivity of the absorption spectrophotometric assay. For $NADP^+$, however, using a fluorescence assay for the appearance of NADPH, it was possible to extend the range of initial substrate concentrations to 2 μM allowing measurement of an apparent K_m of 1.4 μM for $NADP^+$ at saturating aldehyde concentration. Using NAD^+ as the substrate under identical conditions (10^{-4} M dodecanal), an apparent K_m for NAD^+ over 270-fold higher was obtained.

Table 2: Kinetic Properties of Vh.ALDH[a]

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\ \mu M^{-1}$)
acetaldehyde	62600	380	0.006
propanal	2356	610	0.3
butanal	124	560	4
hexanal	44	510	12
dodecanal	3	510	170
NAD^+	390	3660	9
$NADP^+$	1.4	510	364

[a] K_m and k_{cat} with different aldehydes were determined in 50 mM phosphate buffer, 1 mM $NADP^+$, pH 8. K_m and k_{cat} for NAD^+ and $NADP^+$ were determined with dodecanal (10^{-4} M).

Table 2 gives the k_{cat}, K_m, and k_{cat}/K_m values for NAD^+ and $NADP^+$ with dodecanal as substrate. On the basis of the k_{cat}/K_m values, *V. harveyi* aldehyde dehydrogenase functions much more effectively with $NADP^+$ than with NAD^+, with k_{cat}/K_m being 40-fold higher for $NADP^+$.

Determination of the apparent K_m and k_{cat} for Vh.ALDH with different aldehydes at saturating $NADP^+$ concentrations (Table 2) clearly shows that the specificity for aldehydes of longer chain length is reflected in the change in K_m. In contrast, k_{cat} of Vh.ALDH appears to be identical for all aldehydes, indicating that the rate-determining step at saturating substrate concentrations is independent of aldehyde. A detailed kinetic analysis of the native enzyme (Bognar & Meighen, 1983) gave similar results for aldehyde specificity with NAD^+ as substrate and supported a mechanism with a preferred order of addition of NAD^+ before aldehyde and the release of NADH being the last and rate-limiting step. In plots of reciprocal velocity using $NADP^+$ as variable substrate, parallel patterns were obtained with 100 versus 1 μM dodecanal while intersecting patterns were obtained with 100, 250, and 750 μM butanal (data not shown). These patterns were similar to that obtained previously using NAD^+ (Bognar & Meighen, 1983). An apparent dissociation constant for $NADP^+$ of 1.8 μM was calculated (Purich, 1983) from secondary plots of the slopes and intercepts of the reciprocal velocity plots.

To determine if NAD^+ and $NADP^+$ are bound to the same site on the enzyme, assays with both substrates mixed together were conducted. Under conditions where both substrates bind to the same site, the total rate of NAD(P)H production (v) relative to the maximum rate observed with either NAD^+ or $NADP^+$ (i.e., $V_{max(a)}$ with NAD^+) is given by the following equation (Segel, 1993):

$$v/V_{max} = \frac{A/K_a + (V_{max(b)}/V_{max(a)})B/K_b}{A/K_a + B/K_b + 1} \quad (1)$$

with A and B being the concentrations of NAD^+ and $NADP^+$, respectively, and K_a, K_b, $V_{max(a)}$, and $V_{max(b)}$ being the respective K_m's and V_{max}'s. Figure 4 shows a plot of $v/V_{max(a)}$ as a function of the NAD^+ concentration in the presence of fixed concentrations of $NADP^+$ (0, 1.5, and 5 μM). According to eq 1, all of the curves will intersect when the

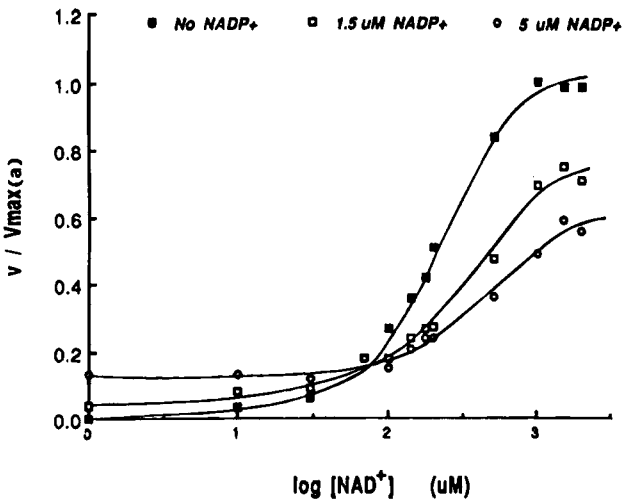

FIGURE 4: Mixed substrate assay. Effect of NAD^+ concentration on the initial velocity of the ALDH reaction at fixed different concentrations of $NADP^+$ (0, 1.5, 5 μM). v/V_{max} is the ratio of each velocity over the V_{max} with NAD^+. All assays were conducted using an absorption assay ($A_{340\ nm}$) for NAD(P)H in 50 mM potassium phosphate buffer, pH = 8, with 0.002% dodecanal (10^{-4} M).

concentration of NAD^+ equals $K_a V_{max(b)}/(V_{max(a)} - V_{max(b)})$, providing that NAD^+ and $NADP^+$ bind to the same site. At this point, the activity is independent of the concentration of $NADP^+$, and it can readily be demonstrated from eq 1 that $v/V_{max(a)}$ is equal to $V_{max(b)}/V_{max(a)}$. In Figure 4, $v/V_{max(a)}$ at the intersection point is 0.16, in close agreement with the ratio of the maximum velocities of NAD^+ and $NADP^+$. Similarly, from the value of the intersect on the abscissa (75 μM) and using $V_{max(b)}/V_{max(a)} = 0.16$, a K_m for NAD^+ of 394 μM can be calculated that is in agreement with the value in Table 2. These results clearly provide strong evidence that NAD^+ and $NADP^+$ bind to the same site on the aldehyde dehydrogenase.

Site-Directed Mutagenesis. Alignment of the amino acid sequence of Vh.ALDH with other aldehyde dehydrogenases (Figure 5) indicates that cysteine 289 of Vh.ALDH corresponds to a conserved cysteine residue found in all aldehyde dehydrogenase (Hempel, 1993). Since Vh.ALDH is $NADP^+$-specific, it is of interest to show whether the conserved cysteine plays a catalytic role as has been proposed for other aldehyde dehydrogenases. The cysteine residue at position 289 was converted to serine (C289S) by site-specific mutagenesis, and the C289S mutant gene was expressed in *E. coli* using T7 polymerase. The 54 kDa mutant ALDH was expressed as well as the lower molecular weight β-lactamase shown in Figure 6 by specific labeling with [^{35}S]-methionine. However, enzyme activity could not be detected in the lysate. Consequently the extract with the C289S mutant was applied to 2′,5′-ADP Sepharose to test the ability of the C289S mutant to bind to this column. As shown in Figure 7A, a radioactive peak was eluted from 2′,5′-ADP Sepharose with $NADP^+$. Resolution of the fractions by SDS−PAGE followed by autoradiography clearly shows the presence of only the 54 kDa mutant Vh.ALDH (Figure 7B). By contrast, labeled β-lactamase had not bound to this column. This result clearly shows that the C289S mutant still retained its conformation and its ability to bind to 2′,5′-ADP. Using this column to purify larger amounts of the C289S mutant to homogeneity (5 mg) allowed analysis for low levels of activity.

Figure 8 compares the activity of the wild type (wt) Vh.ALDH and the C289S mutant in an experiment designed to also measure a pre-steady-state burst of NADPH (Farrés et al., 1994). As shown in Figure 8A, the wild type ALDH incubated at a relatively high protein concentration (0.1 μM) with saturating $NADP^+$ and aldehyde results in a burst of approximately 2 mol of NADPH per mol of enzyme followed by the steady-state production of NADPH with a rate constant at pH 7 of 235 min^{-1}. A pH of 7 was chosen for the reaction rather than the pH for optimum activity to facilitate the extrapolation of NADPH production to zero time. Very little activity was detected with the C289S mutant at 10^{-4} M dodecanal even at the optimum pH and a presteady state burst of NADPH could not be detected. Furthermore, a 3-fold increase in dodecanal concentration with the C289S mutant had no effect on activity. Figure 8B shows that even at a concentration of 3.8 μM, less than 0.01 mol of NADPH is produced per mol of C289S mutant after 5 min. This change corresponds to a maximum of 0.002 min^{-1} for the rate constant.

DISCUSSION

Sequencing of the Vh.ALDH gene has revealed that the gene encodes a protein of 510 amino acids with a molecular weight of 54.5 kDa very close to that reported for Vh.ALDH on the basis of SDS−gel electrophoresis (Bognar & Meighen, 1978). On the basis of a search for proteins with similar sequences, 18.5%−22.5% identity of amino acid sequence was found between *V. harveyi* ALDH and some aldehyde dehydrogenases including betaine aldehyde dehydrogenase from spinach (Weretilnyk & Hanson, 1990), aldehyde dehydrogenase from *E. coli* (Heim & Strehler, 1991), and 2-hydroxymuconic semialdehyde dehydrogenase from *Pseudomonas* (Norlund & Shingler, 1990). Alignment of the sequence of the ALDH from *V. harveyi* with the amino acid sequence of different aldehyde dehydrogenase, shows that only 19 amino acids out of 23 reported invariant residues for 16 different aldehyde dehydrogenases (Hempel et al., 1993) are in the corresponding place in the Vh.ALDH amino acid sequence. On the basis of amino acid sequence homology it has been shown that the sequences of ALDHs share a relatively common core with $NADP^+$ utilizing class 3 ALDHs being N-terminally truncated and C-terminally extended (Hempel et al., 1993), as shown in Figure 5 for two class 3 ALDHs from rat liver. A similar pattern appears to exist for Vh.ALDH, although the extent of N-terminal truncation is less than with class 3 ALDHs. Functionally, the longer N-terminal domains are suggested to be required for tetramer formation (Loomes & Jörnvall, 1991) consistent with Vh.ALDH and other $NADP^+$-utilizing ALDHs, whose structures are known to be dimeric (Evces & Lindahl, 1989; Lindahl et al., 1985; Lindahl & Evces, 1984; Sreerama & Sladek, 1993; Yin et al., 1993).

As has been indicated in Figure 5, Cys_{289} corresponds to Cys_{302} of mammalian class 1 and 2 ALDHs, which has been implicated in a catalytic role (Hempel et al., 1982; von Bahr-Lindström et al., 1985; Blatter et al., 1990; Pietruszko et al., 1991; Wang & Weiner, 1995; Farrés et al., 1995). Although conversion of cysteine 289 of Vh.ALDH to serine using site-directed mutagenesis decreased turnover of the enzyme by at least 5 orders of magnitude, the mutant ALDH retained the ability to bind to 2′,5′-ADP Sepharose, indicating the coenzyme binding site is intact. A pre-steady-state burst of

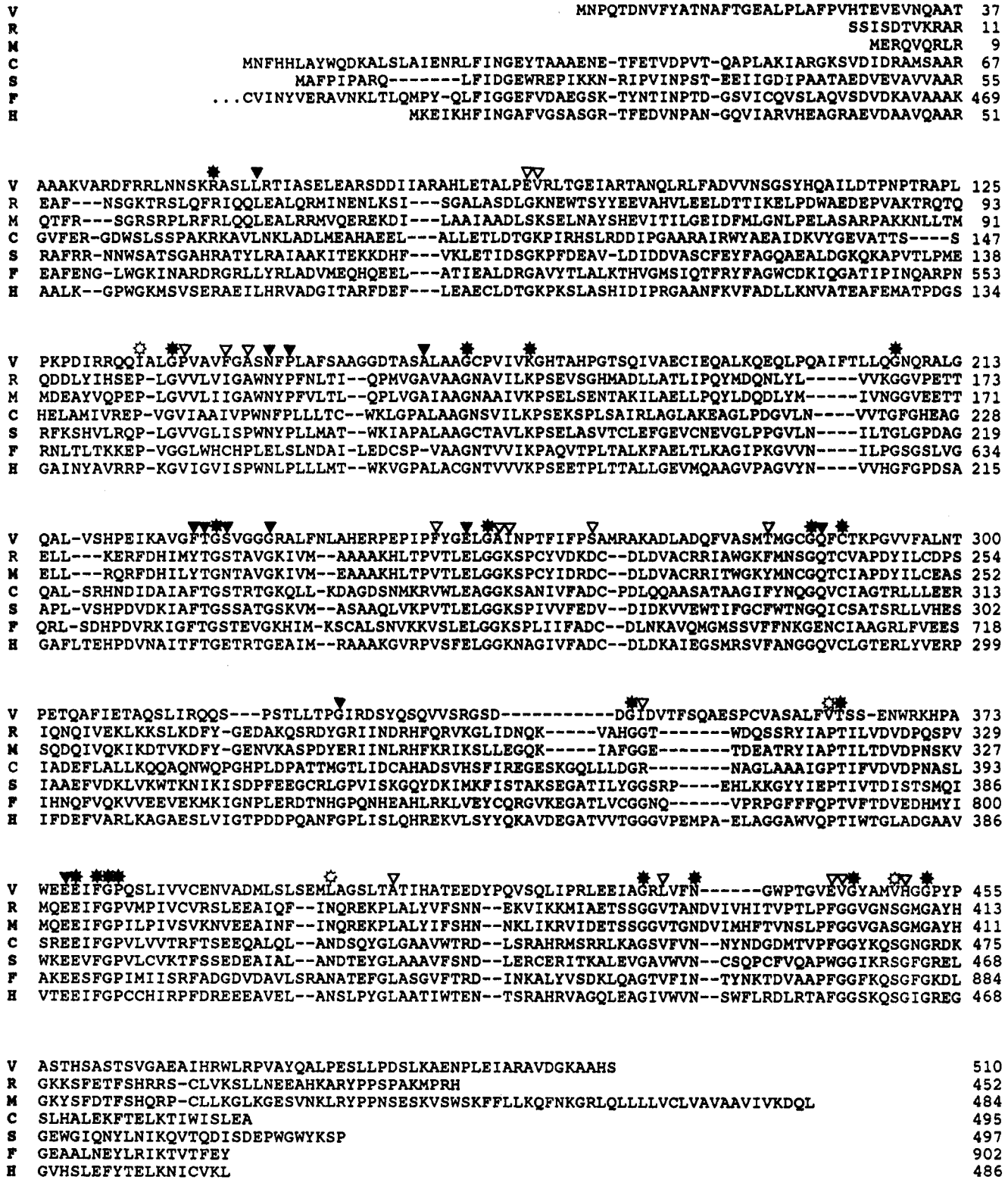

FIGURE 5: Amino acid sequence comparison of the ALDH from *V. harveyi* (V) with ALDHs from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induction of rat liver (class 3) (R), rat liver microsomes (class 3) (M), and *E. coli* (C), betaine aldehyde dehydrogenase from spinach (S), C-terminal domain of rat liver formyl tetrahydrofolate dehydrogenase (F), and hydroxymuconic semialdehyde dehydrogenase from *Pseudomonas* (H). Nineteen out of 23 reported invariant residues and 12 of the 28 reported nearly invariant residues (>87% identity in 16 aldehyde dehydrogenases) can be located in the corresponding places in the Vh.ALDH (filled stars and triangles, respectively). Invariant and nearly invariant residues not conserved in *V. harveyi* ALDH are noted by the empty stars and triangles, respectively. Sequences are from Hempel et al. (1989) (R), Miyauchi et al. (1991) (M), Heim and Strehler (1991) (C), Weretilnyk and Hanson (1990) (S), Cook et al. (1991) (F), and Norlund and Shingler (1990) (H).

NADPH could be observed with the wt Vh.ALDH but not with the C289S mutant implicating the involvement of the thiol group in the pre-steady state. For the native enzyme, the pre-steady-state burst shows that the rate-limiting step must occur after formation of the thiohemiacetal intermediate. The rate-limiting step could either be the deacylation step

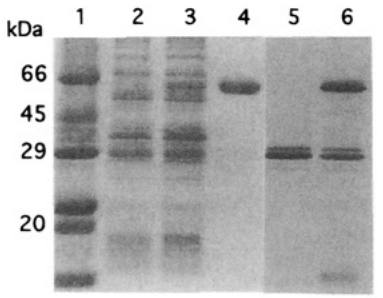

FIGURE 6: Expression of the C289S aldehyde dehydrogenase gene from *V. harveyi* in *E. coli* K38. Transformed *E. coli* K38 contains the pT7-3 plasmid with the C289S aldehyde dehydrogenase gene in reverse (lanes 2 and 5) and forward (lanes 3 and 6) directions with respect to the promoter direction in pT7-3 plasmid. Expression and [^{35}S]methionine labeling were performed as already described in Materials and Methods. The samples were resolved by SDS–PAGE. Lanes 1–4 show protein stained by Coomasie Blue, and lanes 5 and 6 show the autoradiograph of the labeled 54 kDa polypeptide (upper band lane 6) and β-lactamase (lower band). Lanes 4 and 1 are native Vh.ALDH and protein standards, respectively.

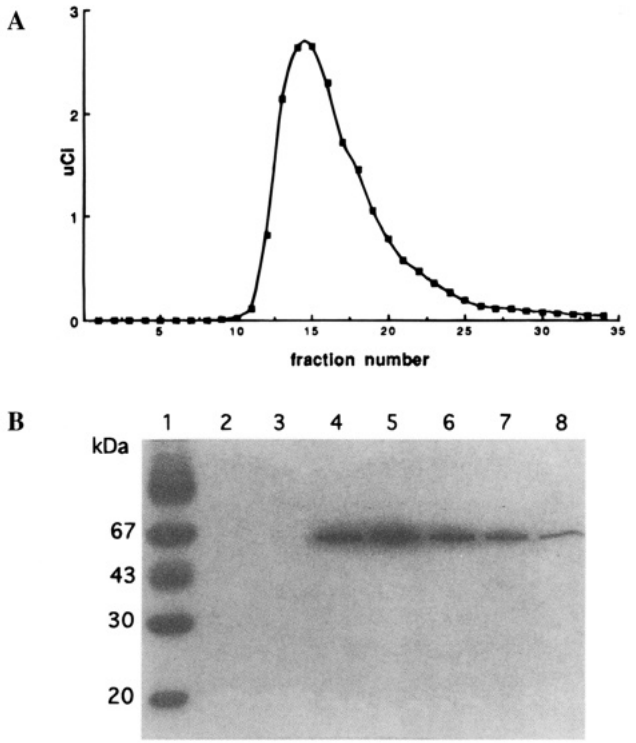

FIGURE 7: Purification of C289S ALDH by 2′,5′-ADP Sepharose. (A) An *E. coli* extract containing [^{35}S]methionine-labeled C289S mutant ALDH (see Material and Methods) was applied to a 1 × 7 cm 2′,5′-ADP Sepharose column, washed with 40 mL of phosphate buffer, and then eluted with 100 μM NADP$^+$ in the same buffer. Fractions (0.8 mL) were collected, and the total radioactivity was reported. (B) Autoradiograph of SDS gel of fractions 9, 11, 13, 15, 17, 19, and 21 (lanes 2–8, respectively). Protein standards stained by Coomasie Blue with the indicated molecular weights are given in lane 1.

or the dissociation of NADPH, but since k_{cat} is much higher with NAD$^+$ than NADP$^+$, it is most likely coenzyme dissociation.

Recently, Farrés et al. (1995) showed that changing the active site cysteine at position 302 to serine in rat liver mitochondrial ALDH did not affect the capability of the mutant enzyme to bind NAD$^+$. Mutation of the active site cysteine to serine eliminated the presteady state burst of NADH and significantly reduced the activity, although the

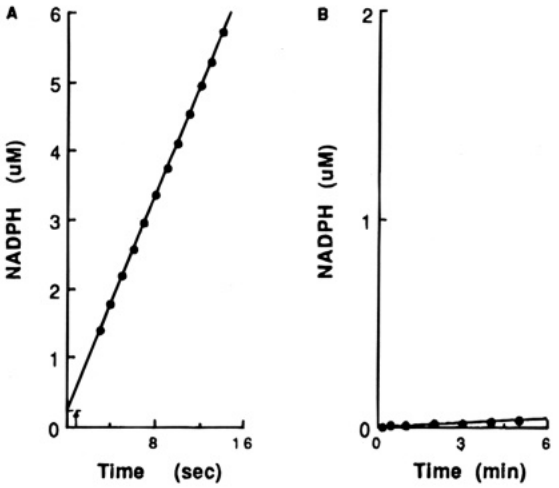

FIGURE 8: Pre-steady-state burst determination of NADPH for Vh.ALDH and C289S. Vh.ALDH (0.1 μM) and C289S (3.8 μM) were incubated with NADP$^+$ (1 mM) in 50 mM potassium phosphate buffer, pH 7 and pH 8, respectively. Fluorescence emission at 460 nm (excitation at 340 nm) was used to establish a base line prior to injection of 3 μL of dodecanal (10^{-2} M) into 300 μL of reaction mixture at zero time. NADPH production was calculated using a standard curve for fluorescence of NADPH at 460 nm, and points from 3 to 14 s for Vh.ALDH and up to 5 min for C289S were plotted (A and B, respectively). The magnitude of the burst for Vh.ALDH at zero time (vertical arrow) corresponds to 2 mol of NADPH per mol (A), while for C289S a burst was not observed.

relative k_{cat} for the C302S mutant was much higher (0.8% of the wt rat liver mitochondrial ALDH) than that observed for the C289S mutant of Vh.ALDH (<0.001% of wt Vh.ALDH). These results indicate that although ALDH from *V. harveyi* is a prokaryotic NADP$^+$-specific aldehyde dehydrogenase, the conserved cysteine residue has a catalytic function, consistent with that reported for mammalian aldehyde dehydrogenases. Interestingly, on the basis of sequence homology, Glu_{253} corresponds to Glu_{268} of human liver aldehyde dehydrogenase reported to be located in the active site (Abriola et al., 1987; Wang & Weiner, 1995) and Gly_{229} and Gly_{234} correspond to Gly_{245} and Gly_{250} of mammalian aldehyde dehydrogenases, which are suggested to be involved in coenzyme binding (Hempel et al., 1984, 1989, 1993; Loomes & Jörnvall, 1991). It should be noted that the Vh.ALDH amino acid sequence from A_{224} through E_{253} provides a reasonable match to that expected for a nucleotide binding site (Wierenga & Hol, 1983) except that the most common motif of GXGXXG is replaced by G_{229}-SVGGG$_{234}$. Comparison of the conserved residues in Vh.ALDH to that in other NAD$^+$-specific aldehyde dehydrogenases along with selected mutagenesis studies should help distinguish those residues involved in the specificity for NADP$^+$ and NAD$^+$.

Although these results show that *V. harveyi* ALDH is part of the superfamily of aldehyde dehydrogenases, its amino acid sequence clearly appears to have diverged the farthest from the consensus sequence for aldehyde dehydrogenases. It is possible to speculate that this sequence divergence may reflect its relatively unique specificity for NADP$^+$. On the basis of the k_{cat}/K_m values, *V. harveyi* ALDH has a 40-fold higher specificity for NADP$^+$ than NAD$^+$. This specificity is unique in that most aldehyde dehydrogenases prefer NAD$^+$ with some aldehyde dehydrogenases having being reported to have a mild preference for NADP$^+$ over NAD$^+$ (Lindahl

& Evces, 1984; Lindahl et al., 1985; Aurich et al., 1987; Evces & Lindahl, 1989; Heim & Strehler, 1991; Sreerama & Sladek, 1993). However, even for these cases, the V_{max}/K_m values for NAD^+ and $NADP^+$ are almost identical with the K_m for $NADP^+$ being higher than for NAD^+. Moreover, the lowest apparent K_m value reported for $NADP^+$ with any aldehyde dehydrogenase is 60 μM (Aurich et al., 1987; Lindahl, 1992). Consequently, the *V. harveyi* ALDH appears to be unique with a K_m of 1.4 μM for $NADP^+$, over 270 times lower than the K_m for NAD^+. Determination of the apparent K_m and k_{cat} for Vh.ALDH with different aldehydes and $NADP^+$ also shows that increasing the aldehyde chain length resulted in a decrease in K_m for aldehyde without a change in k_{cat}, consistent with that previously reported for Vh.ALDH with NAD^+ (Bognar & Meighen, 1978). The unusual $NADP^+$ specificity of the enzyme raises some interesting questions concerning how this enzyme can function in a cellular environment in which the NAD^+ concentration is higher than the NADPH concentration which in turn is expected to be much higher than the $NADP^+$ concentration (Ingraham et al., 1983). It would seem that Vh.ALDH would function most effectively under conditions where oxidized $NADP^+$ is preferentially being produced in order to take advantage of its low K_m for this substrate. This condition may reflect the microenvironment near the fatty acid reductase complex which generates fatty aldehyde for the luminescent reaction along with the concomitant oxidation of NADPH to $NADP^+$ (Riendeau et al., 1982; Rodriguez et al., 1983a,b; Miyamoto et al., 1988). Fatty aldehyde released into the solution and not channeled to the luciferase could readily be removed by the ALDH due to the concurrent release of $NADP^+$ providing an effective safety mechanism to eliminate free aldehyde that could be toxic to the cell.

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