Change of Nucleotide Specificity and Enhancement of Catalytic Efficiency in Single Point Mutants of *Vibrio harveyi* Aldehyde Dehydrogenase†

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ABSTRACT: The fatty aldehyde dehydrogenase from the luminescent bacterium, *Vibrio harveyi* (Vh-ALDH), is unique with respect to its high specificity for $NADP^+$ over NAD^+. By mutation of a single threonine residue (Thr175) immediately downstream of the β_B strand in the Rossmann fold, the nucleotide specificity of Vh-ALDH has been changed from $NADP^+$ to NAD^+. Replacement of Thr175 by a negatively charged residue (Asp or Glu) resulted in an increase in k_{cat}/K_m for NAD^+ relative to that for $NADP^+$ of up to 5000-fold due to a decrease for NAD^+ and an increase for $NADP^+$ in their respective Michaelis constants (K_a). Differential protection by NAD^+ and $NADP^+$ against thermal inactivation and comparison of the dissociation constants of NMN, 2′-AMP, 2′5′-ADP, and 5′-AMP for these mutants and the wild-type enzyme clearly support the change in nucleotide specificity. Moreover, replacement of Thr175 with polar residues (N, S, or Q) demonstrated that a more efficient NAD^+-dependent enzyme T175Q could be created without loss of $NADP^+$-dependent activity. Analysis of the three-dimensional structure of Vh-ALDH with bound $NADP^+$ showed that the hydroxyl group of Thr175 forms a hydrogen bond to the 2′-phosphate of $NADP^+$. Replacement with glutamic acid or glutamine strengthened interactions with NAD^+ and indicated why threonine would be the preferred polar residue at the nucleotide recognition site in $NADP^+$-specific aldehyde dehydrogenases. These results have shown that the size and the structure of the residue at the nucleotide recognition site play the key roles in differentiating between NAD^+ and $NADP^+$ interactions while the presence of a negative charge is responsible for the decrease in interactions with $NADP^+$ in Vh-ALDH.

Aldehyde dehydrogenases (ALDHs)[1] (EC 1.2.1.3) catalyze the $NAD(P)^+$-dependent oxidation of aldehydes and are part of a family composed almost exclusively of NAD^+-dependent enzymes (*1*) although $NADP^+$ can also be used efficiently in a few cases (*2*−*7*). The sequences of over 140 ALDHs have recently been compared (*8*). Only the long-chain aldehyde dehydrogenase isolated from the luminescent bacterium, *Vibrio harveyi* (Vh-ALDH) has a clear preference for $NADP^+$ with a K_m (1.6 μM) 150-times lower and a k_{cat}/K_m (360 min^{-1} μM^{-1}) 40 times higher for $NADP^+$ at saturating dodecanal than the same parameters with NAD^+ as coenzyme (*9*−*11*). The strong specificity of Vh-ALDH for $NADP^+$ is very advantageous for investigating the function and structure of ALDHs as the recombinant enzyme can be readily purified to homogeneity from crude extracts in a single step by affinity chromatography on 2′5′-ADP Sepharose.

The crystal structures of three ALDHs have been recently reported; class 2 bovine mitochondrial ALDH (ALDH2) (*12*), class 3 rat ALDH (ALDH3) (*13*), and class 9 betaine ALDH from cod liver (ALDH9) (*14*). All three ALDHs are NAD^+ specific and have about 20−25% sequence identity with Vh-ALDH. Interestingly, the recognized binding motif for these ALDHs is the β_D-α_D(β_B) sequence in the Rossmann fold rather than the classical β_A-α_A-β_B binding motif found for other oxidoreductases (*13*). A glutamic acid residue has been recognized in both the rat ALDH3 (Glu140) and bovine ALDH2 (Glu195) crystal structures as bonding to the 2′-hydroxyl of the adenosine ribose and appears to serve as part of the NAD^+ recognition site (*13*, *12*). The glutamic acid residue is immediately after the β_B strand in the Rossmann fold in the same location as the negatively charged nucleotide recognition sites found in other NAD^+-dependent enzymes (*15*). In contrast, cod liver ALDH9 has a neutral proline at this site and a nearby lysine residue interacts with the 2′-oxygen of the adenosine ribose of NAD^+, indicating that this site is not always involved in nucleotide interaction (*14*).

Alignment of the sequence of Vh-ALDH with other ALDHs shows that a threonine residue (Thr175) is at the site for recognition of the substituent at the 2′-hydroxyl of adenosine in $NAD(P)^+$ in contrast to glutamic acid in rat ALDH3 (*13*) and bovine ALDH2 (*12*) and proline in cod liver ALDH9 (*14*). The presence of threonine at this point

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[1] Abbreviations: T175D, T175E, T175S, T175N, and T175Q, mutant aldehyde dehydrogenases where the residue at one position is replaced by another; NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; ALDH, aldehyde dehydrogenase; SDS, sodium dodecyl sulfate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; NMN, nicotinamide mononucleotide.

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could reflect the different coenzyme specificity for Vh-ALDH (*9*, *11*) as other oxidoreductases which can function effectively with $NADP^+$ have a neutral or polar residue rather than a negatively charged residue at this position (*15*).

In the present investigation, Thr175 of Vh-ALDH has been mutated to glutamic acid, aspartic acid, serine, asparagine, and glutamine and each of the mutants purified to homogeneity, and the kinetic properties characterized. Remarkably, mutants T175S, T175Q, and T175N all had enhanced activities for NAD^+ in terms of k_{cat}/K_m without a significant loss in $NADP^+$ activity. Both the T175D and T175E mutants strongly preferred NAD^+ with the T175E mutant having a preference for NAD^+ over $NADP^+$ at saturating dodecanal 5000-fold higher than that for the wild-type enzyme. These results show that this site is of critical importance in designating coenzyme specificity even in ALDHs in which the binding motif for $NAD(P)^+$ differs from the classical β_A-α_A-β_B motif in the Rossmann fold found at the nucleotide binding site in other oxidoreductases.

MATERIALSAND 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. $NADP^+$ and NAD^+ were purchased from Sigma Chemical Co. Aldehydes were from Aldrich. The bacterial strains used in these studies were *Escherichia coli* K38 and *Vibrio 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 (*16*). [^{35}S]Methionine labeling was performed as described before (*9*). 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, and 10 mM 2-mercaptoethanol and lysed by sonication. 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. All protein samples except the T175E mutant were applied to a column of adenosine 2′,5′-bisphosphate (2′,5′-ADP) linked to Sepharose (Pharmacia Biotech) equilibrated in 50 mM phosphate buffer, pH 7. The column was washed with 10−50 times the column volume of the above-mentioned buffer and eluted with 100 μM $NADP^+$ in the same buffer. The T175E mutant was loaded onto a column of NAD^+ linked to agarose (Sigma Chemical Co.) and eluted with 1 mM NAD^+ in 50 mM phosphate buffer, pH 7. The eluted enzyme fractions were pooled and concentrated by dialysis against 30% glycerol, 50 mM sodium potassium phosphate, pH 7, and 10 mM 2-mercaptoethanol.

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 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 was 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 Grafit to provide the best fits and standard errors. Inhibition constants for nucleotide analogues were obtained from Dixon plots with heptanal at saturating concentration (340 μM) and variable concentrations of inhibitors at different fixed concentrations of NAD^+.

Site-Directed Mutagenesis. The *V. harveyi* ALDH gene in M13 was mutated based on the method described by Kunkel (*17*) using the Muta-Gene M13 in Vitro Mutagenesis kit from Bio-Rad. The codon for threonine at position 175 (ACT) was altered to GAT (Asp), GAA (Glu), TCT (Ser), 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 (*18*).

Thermal Stability. For thermal inactivation, the enzymes were incubated at 37 °C in 50 mM phosphate, pH 7, containing 10 mM 2-mercaptoethanol in the presence or absence of $NAD(P)^+$. At different times, aliquots from each sample were taken and assayed for dehydrogenase activity.

RESULTS

Mutation, Expression, and Purification of V. harveyi ALDH. Alignment of the sequence of Vh-ALDH with the β_A-α_A-β_B sequence of the Rossmann fold in ALDHs with known crystal structures (ALDH2, ALDH3 and ALDH9) is shown in Figure 1. In contrast to the negatively charged residue (Glu) found just downstream of the β_B strand in ALDH2 and ALDH3 and believed to be responsible for recognition of the 2′-hydroxyl of NAD^+, Vh-ALDH contains a threonine residue (Thr175). Although this result is consistent with a preference for $NADP^+$ by Vh-ALDH, it should be noted that the NAD^+-dependent cod liver ALDH9 contains a neutral proline residue at this position (*14*).

To demonstrate that the threonine residue at position 175 may be critical for the $NADP^+$ specificity of Vh-ALDH, this residue was mutated to glutamate and aspartate as well as to serine, asparagine, and glutamine by site-specific mutagenesis. The mutated ALDH gene was expressed to high levels in *E. coli*, and assays in crude extracts indicated that


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VH   ALDH   GPVAVFGASNFPLAFSAAGGDTASALAAGCPVIVKGHTAHP
bov ALDH2   GVCGQIIPWNFPLL  MQAWKLGPALATGNVVVMKVAEQTP
rat ALDH3   GVVLVIGAWNYPFN  LTIQPMVGAVAAGNAVILKPSEVSG
cod ALDH9   GVCAGILAWNYPFM  IAAWKCAPALACGNAVVFKPSPMTP
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FIGURE 1: Alignment of the sequences of Vh-ALDH and lung carbonyl reductase with the β_A-α_A-β_B sequences of the Rossmann folds of bovine ALDH2, rat ALDH3, and cod liver ALDH9. Residues identical to those in Vh-ALDH are given in bold. The boxed N gives the position of the amino acid in the recognition site for the moiety at the 2′-hydroxyl of the adenosine ribose in NAD(P$^+$) with the corresponding amino acids in the ALDHs underlined and given in bold.

all the mutated enzymes had retained high activity but T175D and T175E had altered nucleotide specificity.

Purification of the wild-type Vh-ALDH to homogeneity was accomplished in a single step by affinity chromatography on 2′,5′-ADP Sepharose and elution with NADP$^+$ (Figure 2a). Similar purification profiles (not shown) were obtained for the T175S, T175N, and T175Q mutants. The T175D mutant could also bind to the 2′5′-ADP Sepharose and be purified to homogeneity while all the activity associated with the T175E mutant passed through the column before elution with NADP$^+$, indicating that its affinity for 2′5′-ADP and hence NADP$^+$ had been greatly reduced (Figure 2a). However, it was possible to purify the T175E mutant to homogeneity in a single step on a NAD$^+$-Sepharose column with the NAD$^+$ linked to the column via the N-6 nitrogen of the adenine ring (Figure 2b). Other NAD$^+$ Sepharose columns with the NAD$^+$ linked via the C-8 carbon of the adenine ring or the ribose hydroxyls did not retain the T175E mutant, indicating that the backbone support in these cases interferes with interaction of the NAD$^+$ ligand with the enzyme. SDS gel electrophoresis of the purified enzymes show that all the mutated proteins gave one major band of molecular mass of about 50−55 kDa. Small amounts of contaminants (<2%) can be detected in the T175S mutant when large amounts of protein (∼10 μg) are analyzed on the SDS gel (Figure 2c).

Kinetic Properties and Aldehyde Dehydrogenase Activity. The kinetic properties and nucleotide specificity of the mutants were initially characterized using dodecanal at saturating concentrations as substrate (Table 1). All mutants retained high turnover rates with both NAD$^+$ and NADP$^+$; k_{cat} with NAD$^+$ remained the same as the wide-type enzyme except for a 2.5-fold increase for the T175Q mutant, while k_{cat} with NADP$^+$ increased about 3-fold for all mutants except for T175S, whose k_{cat} decreased 2-fold.

The major effect arising from mutation of threonine 175 was an alteration in the respective apparent K_ms for NAD$^+$ and NADP$^+$. For the T175E mutant, the K_m for NADP$^+$

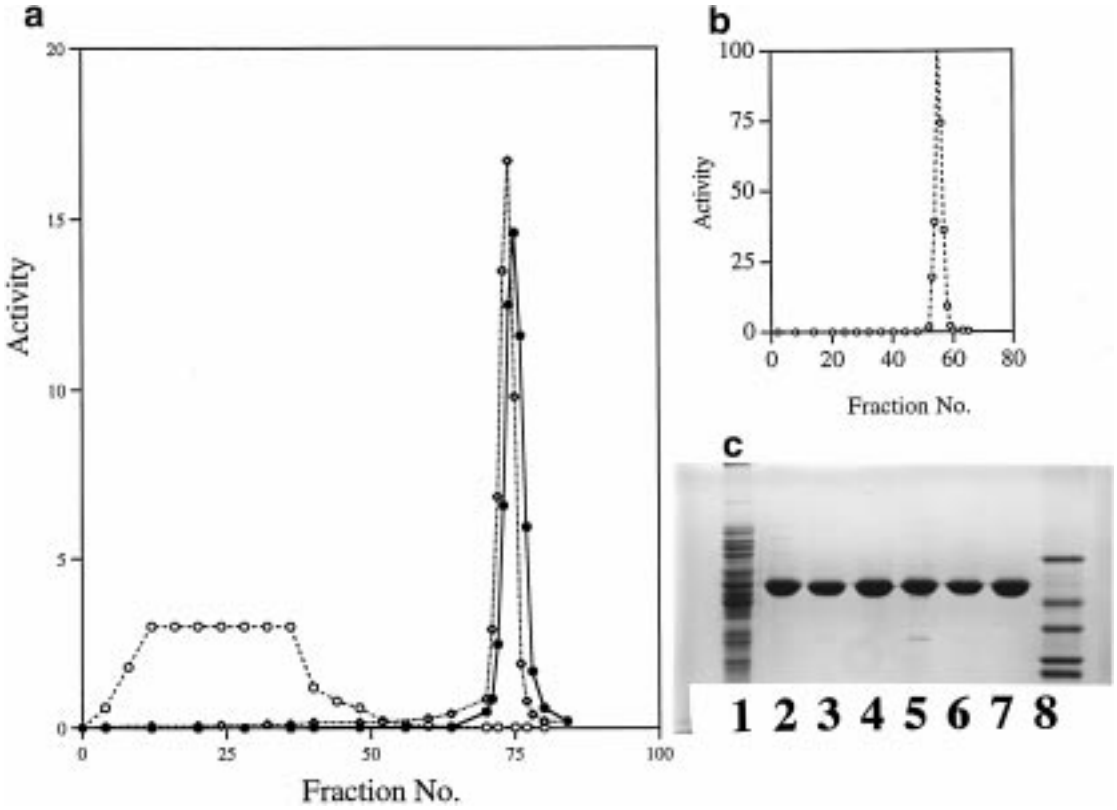

FIGURE 2: Purification of *V. harveyi* ALDH mutants. (A) Plot of the activity (μmol/min/ml) of extracts (50 mL) of Vh-ALDH (●), T175D mutant (◇), and T175E (○) mutant applied to a 1 × 10 cm column of 2′5′-ADP Sepharose equilibrated in 0.05 M phosphate, pH 7.0, washed with 100 mL of the same buffer and then eluted with 100 μM NADP$^+$ in 0.05 M phosphate, pH 7.0, at 4 °C. Fractions of 1.6 mL were collected and assayed for NAD$^+$-dependent ALDH activity. (B) Elution profile of an extract of the T175E mutant applied to a l × 10 cm column of NAD$^+$ Sepharose equilibrated in 0.05 M phosphate, pH 7, washed with 100 mL of the same buffer and then eluted with 1 mM NAD$^+$ in 0.05 M phosphate, pH 7, at 4 °C. SDS gel electrophoresis of the purified ALDH mutants. Ten micrograms of protein was applied to each lane except for the crude extract (50 μg of protein, lane 1). Lanes 2−8 contain the wild-type enzyme, T175D, T175E, T175S, T175N, T175Q, and protein standards, respectively.

Table 1: Kinetic Parameters for Native 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	382 ± 20	3400 ± 89	9 ± 0.5	1.6 ± 0.3	550 ± 30	330 ± 64
T175D	124 ± 12	3000 ± 68	24 ± 2.4	260 ± 34	1700 ± 80	6.6 ± 0.9
T175E	56 ± 3	3200 ± 36	57 ± 3	3500 ± 80	1550 ± 180	0.44 ± 0.05
T175S	127 ± 10	2900 ± 51	23 ± 1.9	2.2 ± 0.1	260 ± 3	120 ± 6
T175N	194 ± 24	3800 ± 92	20 ± 2.5	17 ± 1.6	1480 ± 40	87 ± 8
T175Q	50 ± 3	8700 ± 83	173 ± 10	3.8 ± 0.6	1390 ± 50	370 ± 60

[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).

Table 2: Increase in Nucleotide Specificity for NAD$^+$ on Mutation of Thr 175

enzyme	change in K_m (NAD$^+$)/ K_m (NADP$^+$)[a]	change in V_m/K_m (NAD$^+$)/ V_m/K_m (NADP$^+$)[b]
T175D	0.002	130
T175E	0.000 07	5000
T175S	0.25	7
T175N	0.05	8
T175Q	0.06	17

[a] Ratio of K_m (NAD$^+$)/K_m (NADP$^+$) of mutants over that for the wild-type enzyme with saturating dodecanal. [b] Ratio of V_m/K_m (NAD$^+$)/ V_m/K_m (NADP$^+$) of mutants over that for the wild-type enzyme with saturating dodecanal.

increased by 2000-fold and the K_m for NAD$^+$ decreased by 7-fold, resulting in a 14000-fold drop in the ratio of K_m-(NAD$^+$)/K_m(NADP$^+$) for the mutant compared to the wild-type enzyme (Table 2). A lower but similar effect was observed with the T175D mutant with the K_m for NADP$^+$ increasing by 160-fold and the K_m for NAD$^+$ decreasing by 3-fold. Replacement of T175 with uncharged residues also decreases the K_m for NAD$^+$ with only small increases in the K_m for NADP$^+$. Small changes (2−3-fold) can also be observed in k_{cat}. Particularly noteworthy is the 20-fold increase in catalytic efficiency in terms of k_{cat}/K_m for the T175Q mutant without any loss in activity with NADP$^+$. On the basis of k_{cat}/K_m as a measure of specificity for the respective nucleotide, the relative preference of Vh-ALDH for NAD$^+$[V_m/K_m(NAD$^+$)]/[V_m/K_m(NADP$^+$)] increased by 5000-fold on mutation of threonine 175 to glutamic acid with smaller increases (7−130-fold) on replacement of threonine 175 with other residues (Table 2).

As the apparent K_m for dodecanal is too low to allow a complete kinetic analyses, the effects of changing aldehyde concentration on activity in a two-substrate were studied with a shorter chain aldehyde, heptanal. Table 3 gives the Michaelis constants for the nucleotide (NAD$^+$ or NADP$^+$) and heptanal substrates and the k_{cat} values for each of the mutants derived from secondary plots of the intercepts and slopes of the primary Lineweaver−Burk plots. As the slope of the Lineweaver−Burk plots were parallel or very close to parallel with all the mutants with either NAD$^+$ or NADP$^+$ as substrate, the K_{ab} term, which reflects the dissociation constant for the first substrate in an ordered mechanism, could not be determined (*10*). Attempts to measure NADPH binding directly by fluorescence were unsuccessful as no significant differences were observed on mixing with the enzyme.

The Michaelis constants for NAD$^+$ and NADP$^+$ (K_a) for the mutant and wild-type ALDHs with heptanal as substrate

Table 3: Kinetic Constants for Native and Mutant Aldehyde Dehydrogenases[a]

	NAD$^+$				
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})
WT	205	190	4800	23	25
T175D	120	330	8600	72	26
T175E	58	280	10 900	190	39
T175S	130	160	6100	47	38
T175N	97	170	5400	55	32
T175Q	31	99	9500	300	96
	NADP$^+$				
enzyme	K_a (NADP$^+$) (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})
WT	1.2	8.6	530	440	62
T175D	260	140	7100	27	51
T175E	3300	520	9700	3.0	19
T175S	1.4	8.1	880	630	109
T175N	6.8	6.8	2000	290	290
T175Q	8.4	14	2000	240	143

[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 the 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 2 and 18% of the indicated values.

were similar to the apparent K_m values for the respective nucleotides with saturating dodecanal (Table 1), indicating that the interaction with the nucleotide substrate was not affected by the chain length of the aldehyde substrate. The turnover rates (k_{cat}) for the wild-type ALDH and the T175N and T175Q mutants with heptanal were comparable to the k_{cat} values with dodecanal with the same nucleotide. For the T175D, T175E, or T175S mutants, the turnover rates with heptanal were about three times higher than the respective turnover rates with dodecanal. The Michaelis constants for heptanal (K_b) for the mutants generally remained within 2-fold of that for the wild-type ALDH with the respective nucleotide except for the T175D and T175E mutants with NADP$^+$ as coenzyme.

Inhibition with Nucleotide Analogues. Addition of various nucleotide analogues resulted in inhibition of the enzyme activity. From Dixon plots ($1/v$ vs I at variable NAD$^+$ concentrations) with the wild-type enzyme and the T175E and T175D mutants, dissociation constants for the inhibitors (K_i) were measured for 2′,5′-ADP, 2′-AMP, 5′-AMP, and NMN (Table 4). The dissociation constants for NMN binding to the wild-type enzyme and the T175E and T175D mutants were almost identical. The dissociation constants for 5′-AMP were 2- and 8-fold lower for the T175D and T175E mutants,

Table 4: Inhibition Constants for Nucleotide Analogues[a]

	K_i (mM)		
inhibitor	WT	T175D	T175E
2′5′ADP	0.05	1.0	>10
2′AMP	1.0	2.3	11.4
5′AMP	4.7	2.3	0.6
NMN	3.6	3.1	5.2

[a] Inhibition constants were measured from Dixon plots as described in Materials and Methods.

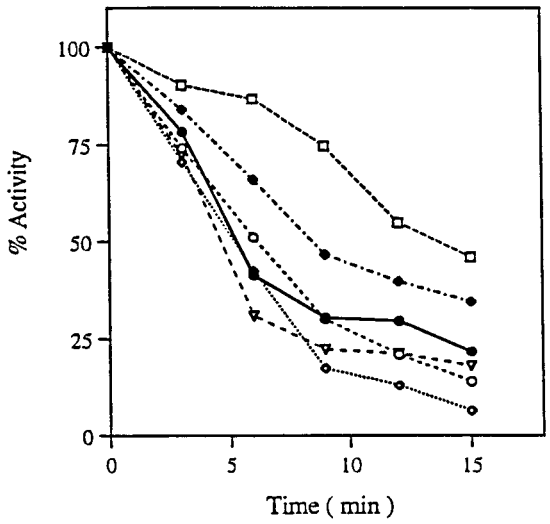

FIGURE 3: Thermal inactivation of the wild-type Vh-ALDH and T175 mutants at 37 °C in 50 mM phosphate, pH 7.0. WT (●), T175D (◇), T175E (○), T175S (□), T175N (▽), T175Q (◆).

respectively, compared to the K_i for the wild-type enzyme. This decrease almost exactly parallels the decrease in K_m for NAD^+ for the two mutants relative to that for the wild-type enzyme. In contrast, the dissociation constants for 2′-AMP and in particular 2′,5′-ADP were much higher for the two mutants than the respective K_i for the wild-type enzyme.

Thermal Stability and Nucleotide Protection. The thermal labilities of the Thr175 mutants at 37 °C were very similar to that of the wild-type enzyme except for the more stable T175Q and T175S mutants (Figure 3). Addition of nucleotides stabilized both the wild-type enzyme and the mutants against thermal inactivation as shown in Figure 4 for the wild-type enzyme and the T175E mutant. Addition of 0.2 (or 0.02) mM $NADP^+$ to the wild-type enzyme affords strong protection against thermal inactivation while addition of NAD^+ only provides protection to a very small extent. In contrast, the nucleotide specificity in terms of protection against thermal inactivation is the reverse with the T175E mutant. $NADP^+$ provides little if any protection of T175E against thermal inactivation while 0.02 mM NAD^+ provides good protection and 0.2 mM NAD^+ completely stabilizes the T175E mutant against thermal inactivation at 37 °C. The differential protection against thermal inactivation by $NADP^+$ and NAD^+ for the wild-type and T175E mutant provides clear support that the mutation of threonine 175 to a glutamic acid residue causes the nucleotide specificity of Vh-ALDH to change from a $NADP^+$-specific to a NAD^+-specific dehydrogenase due to a preferred binding of NAD^+.

pH Dependence. Replacement of Thr175 by serine, asparagine, or glutamine had very little effect on the dependence of dehydrogenase activity on pH (Figure 5a). The decrease in activity of the wild-type ALDH at higher pH reflected primarily an increase in K_m for NAD^+, which was partially compensated by an increase in k_{cat} (data not shown). The tendency for the activity of the T175S, T175N, and T175Q mutants to be higher than that of the wild-type enzyme at higher pH appears to correlate with their lower K_ms for NAD^+.

A strong shift to higher pH for maximum activity could be observed with mutants in which a negatively charged residue (T175D, T175E) is introduced (Figure 5b). The increase in activity from pH 8 to 9 for these mutants represents primarily an increase in k_{cat} as assays were conducted at concentrations of NAD^+ (1.5 mM) above the K_m at both pHs. The K_ms for NAD^+ at pH 9 with the T175E and T175D mutants (with saturating dodecanal) increased about 10-fold (to 0.6 mM and 1.3 mM, respectively) compared to the values at pH 8 while k_{cat} values increased between 3 and 4-fold. It should be noted that in terms of k_{cat}/K_m optimum activity still occurs at pH 8 for these mutants.

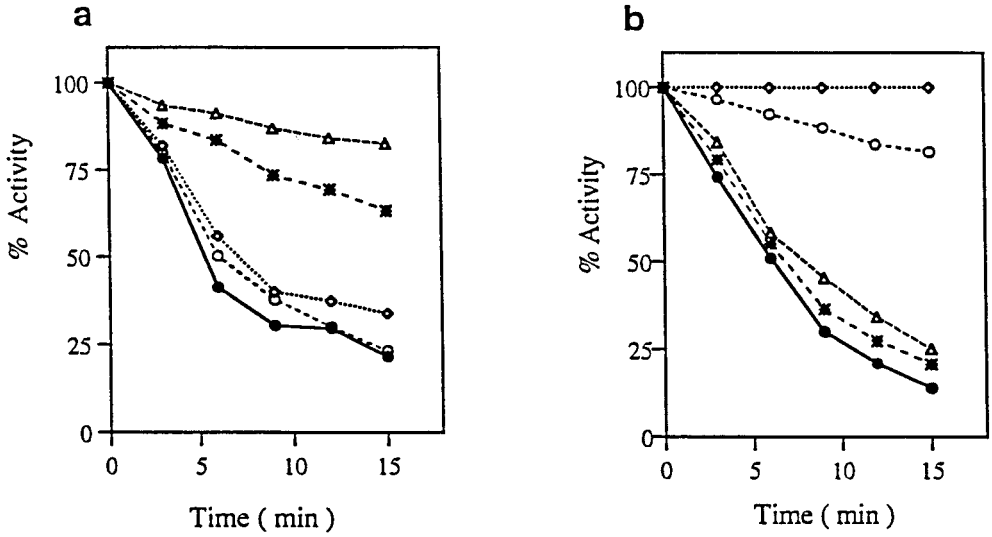

FIGURE 4: $NAD(P)^+$ protection against thermal inactivation of wide type and T175E ALDH. The wild-type Vh-ALDH(a) and the T175E mutant(b) were incubated at 37 °C in 50 mM phosphate, pH 7.0, with the indicated concentrations of $NAD(P)^+$. Without cofactor (●), with 200 μM NAD^+ (◇), with 20 μM NAD^+ (○), with 200 μM $NADP^+$ (△), with 20 μM $NADP^+$ (*).

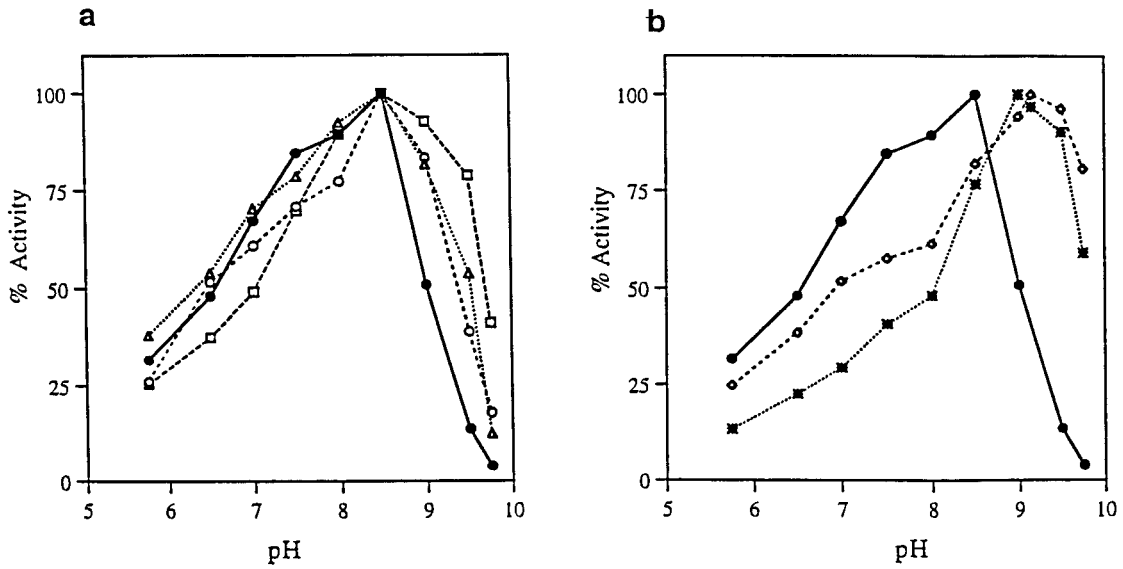

FIGURE 5: Dependence of the NAD^+-dependent aldehyde dehydrogenase activity of Vh-ALDH mutants on pH. Activities were measured in 50 mM Tris-chloride at the indicated pH with 1.5 mM NAD^+ and 100 uM dodecanal. Identical activities were obtained at pH 8 in the standard 50 mM phosphate buffer. (a) WT (●), T175S (△), T175N (○), and T175Q (□); (b) WT (●), T1175D (*), and T175E (◇).

DISCUSSION

The present work has shown that by mutation of only a single residue, Thr175, the nucleotide specificity of Vh-ALDH changed from $NADP^+$ to NAD^+. The primary effect of the mutation on the enzyme was to change the apparent K_m or Michaelis constant (K_a) for the respective nucleotide with smaller effects on the rates of catalytic turnover. Replacement of threonine with a negatively charged residue, glutamic or aspartic acid, caused the largest effects. For the T175E mutant, the K_m (or K_a) increased by over 2000-fold for $NADP^+$ and decreased by 4−7-fold for NAD^+ with heptanal or dodecanal as substrate. Coupled with the changes in k_{cat}, the specificity for NAD^+ relative to $NADP^+$, $[k_{cat}/K_m(NAD^+)]/[k_{cat}/K_m(NADP^+)]$ increased by 1200−5000-fold depending on the particular long-chain aldehyde. Replacement of threonine with polar residues (N, S, and Q) had smaller effects upon the nucleotide specificity of Vh-ALDH but still increased the specificity for NAD^+ by 7−17-fold. In all cases, replacement of threonine decreased the K_a for NAD^+ and increased the ability of the ALDH to interact with NAD^+. Indeed, replacement of threonine with the larger residue, glutamine, caused a decrease in K_a for NAD^+ (∼7-fold) identical to that found for T175E while replacement of Thr175 with aspartate, asparagine, or serine also decreased the K_a for NAD^+ but to a much smaller extent. The catalytic properties of the T175Q mutant were quite unique in that the catalytic efficiency with NAD^+ was increased 15−20-fold while the activity (k_{cat}/K_m) with $NADP^+$ remained between 50 and 110% of that of the wild-type enzyme. An increase in catalytic efficiency on mutation without a negative affect on other kinetic properties is a relatively rare event. These results would indicate that interactions with the 2′-hydroxyl of NAD^+ are equally effective with polar and charged residues and that a larger amino acid side chain is necessary for optimum interactions presumably to occupy the space vacated by the 2′-phosphate of $NADP^+$. Although glutamate disrupts the interactions with $NADP^+$, an uncharged amino acid of similar size (i.e., Q) only increases K_m to a small degree for $NADP^+$. Moreover, this effect is partially compensated by an increase in k_{cat}, consistent with dissociation of $NADP^+$ being the rate-limiting step in the reaction.

Just after completion of these experiments, the crystal structure of Vh-ALDH with bound $NADP^+$ was elucidated (Ahvazi et al., manuscript in preparation). Although the location of the nicotinamide ring could not be determined presumably due to the high mobility of the ring in the crystal structure, the position of the adenosine 2′-phosphate moiety could be clearly defined. Figure 6a shows the region of Vh-ALDH immediately in contact with the 2′-phosphate and the close interaction with Thr175. Replacement of Thr175 with glutamic acid (or glutamine) puts the carboxyl group (or the amide group) of glutamic acid (glutamine) in a highly favorable position to hydrogen bond to both the 2′- and 3′-hydroxyls of adenosine in the NAD^+ moiety (Figure 6b). Moreover, without introduction of any steric clashes in the Vh-ALDH crystal structure, a small change in the torsional angles of glutamine places the amide group in a very good position to maintain contacts with the phosphate of $NADP^+$ (Figure 6c).

The structures in Figure 6 show that introduction of a residue with a different size and structure (i.e., glutamine) would enhance the interactions with NAD^+ and at the same time not disrupt the interactions with $NADP^+$. As replacement of Thr175 with glutamic or aspartic acid caused large increases in the k_{cat}/K_m for $NADP^+$ while replacement with glutamine and asparagine had little effect on the k_{cat}/K_m for $NADP^+$, it would suggest that electrostatic repulsions are the primary reason for disruption of $NADP^+$ binding with the larger group (i.e., glutamic acid) being more effective. Perhaps of greater significance is the recognition that a mutant with glutamine at the nucleotide recognition site can function equally well with NAD^+ or $NADP^+$ [i.e., k_{cat}/K_m (or K_a) for $NADP^+$ and NAD^+ are within 2-fold of each other with heptanal or dodecanal]. This may explain why a small residue with more restricted torsional angles due to a β-methyl group (i.e., threonine) would be the preferred residue at the nucleotide recognition site to generate a $NADP^+$-specific ALDH. Not only would threonine interact well with $NADP^+$ but it would

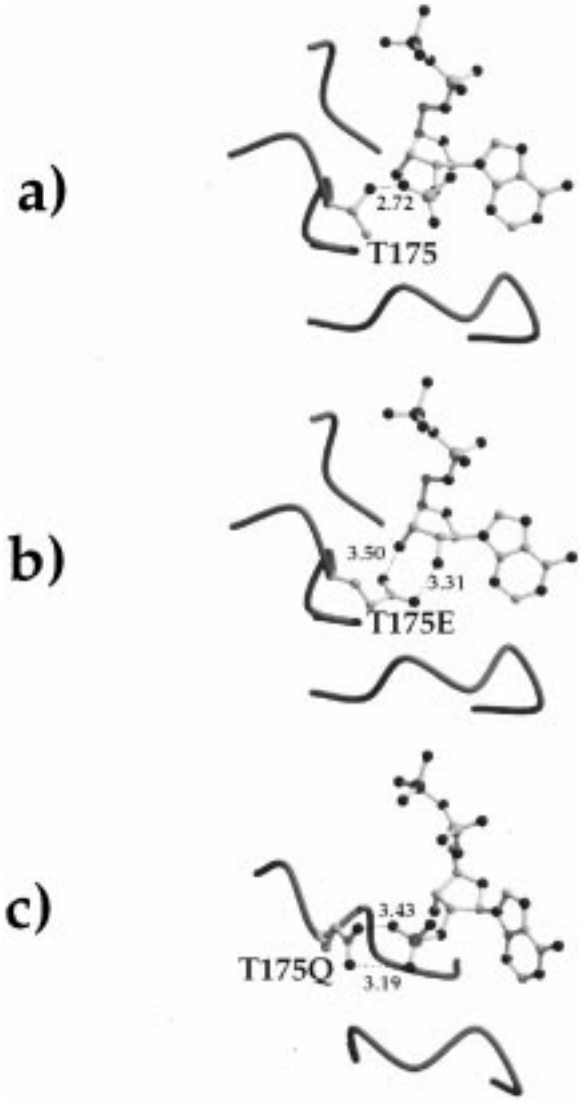

FIGURE 6: The structure of *V. harveyi* aldehyde dehydrogenase in the region of the adenine ribose moiety of the cofactor. (a) The wild-type enzyme with a threonine at position 175 shown to interact with the 2′-phosphate of the $NADP^+$ cofactor. (b) A model showing the T175E mutant bound to the NAD^+ cofactor. (c) A model showing the T175Q mutant bound to the $NADP^+$ cofactor. Small changes in the torsion angles (used in panel b) without introducing steric clashes in the Vh-ALDH crystal structure permit strong interaction with $NADP^+$.

have much less flexibility than other polar residues to adapt to a different configuration to bind in an optimal manner with NAD^+. In this regard, a NADPH-specific carboxyl reductase has also been recently shown to have a threonine residue at the nucleotide recognition site (*19*).

Measurement of the dissociation constants (K_i) of four inhibitors (NMN, 2′-AMP, 5′-AMP, and 2′5′-ADP) with T175D, T175E, and the wild-type enzyme provided additional support for the role of Thr175 in interacting with the 2′-phosphate of $NADP^+$. The dissociation constant for NMN was not affected by mutation of T175, consistent with this residue interacting with the 2′ position of the ribose of adenosine. The K_i for 5′-AMP was decreased rather than increased on mutation of Thr175 with the mutant with the largest substituent (T175E) having the greatest effect, a result which closely parallels the effects on the $K_a(K_m)$ for NAD^+. Similarly, the K_i values were higher for 2′-AMP and 2′5′-ADP with the T175D and T175E mutants than the respective K_i values with the wild-type enzyme and comparable to the changes in $K_a(K_m)$ for $NADP^+$. Interestingly, the effect of replacement of T175 by a charged residue has a much greater effect on binding of 2′5′-ADP than on 2′-AMP binding. This result combined with the low K_i for 2′5′-ADP(50 ?M) compared to 1 mM for 2′-AMP and 4.7 mM for 5′-AMP for the wild-type Vh-ALDH indicates that interaction of both phosphates with the enzyme is critical for tight binding of $NADP^+$. Indeed mutation at Thr175 could readily shift the position of the ribose and cause the interactions with the 5′-phosphate to be altered in $NADP^+$(and 2′5′-ADP) without disrupting the interactions with the 5′-phosphate of NAD^+ (and 5′-AMP).

The change of the nucleotide specificities of NAD(P) (H)-dependent oxidoreductases has been the focus of the research of a number of laboratories (*15*, *19*, *20*, *21*). In most cases, multiple residues in the active site were altered to produce a change in specificity, although alteration of the residue interacting with the 2′-hydroxyl or phosphate of the ribose of adenosine in NAD(P) (H) had the most significant effect. Substitution of two residues in $NADP^+$-dependent isocitrate dehydrogenase, including replacement of a lysine by an aspartate which interacts with the adenosine ribose, increased the preference toward NAD^+ by about 6000-fold. However, the change in specificity arose exclusively due to a loss in activity with $NADP^+$ with the double mutant having similar k_{cat}/K_m values for NAD^+ and $NADP^+$. Additional mutations were then necessary to improve the catalytic activity of isocitrate dehydrogenase with NAD^+ providing an excellent demonstration of protein engineering (*21*).

For most oxidoreductases, the nucleotide coenzyme interacts with the classical β_A-α_A-β_B motif in the Rossmann fold. In contrast, in the crystal structures of rat ALDH3 and bovine ALDH2, the nucleotide interacts with a β_D-$\alpha_D(\beta_B)$ motif composed of the β_D-α_D loop and the β_B strand (*12*−*14*). The recent elucidation of the crystal structure of *V. harveyi* ALDH with bound $NADP^+$ has also demonstrated that the nucleotide interacts with the β_D-$\alpha_D(\beta_B)$ rather than the β_A-$\alpha_A(\beta_B)$ motif (Ahvazi et al. manuscript in preparation). A negatively charged residue (glutamic acid) located just after the β_B strand is in close proximity with the ribose hydroxyls of the adenosine in NAD^+ in both ALDH2 and ALDH3. The current studies clearly show that a single amino acid located immediately downstream of the β_B strand (Thr175) plays a key role in controlling nucleotide specificity in Vh-ALDH. These results indicate that the β_B strand and the downstream nucleotide recognition site may be conserved in all NAD(P) (H)-dependent enzymes with a Rossmann fold even with different motifs for nucleotide binding.

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