Isolation and Characterization of an Aldehyde Dehydrogenase Encoded by the *aldB* Gene of *Escherichia coli*†

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An aldehyde dehydrogenase was detected in crude cell extracts of *Escherichia coli* DH5 α . Growth studies indicated that the aldehyde dehydrogenase activity was growth phase dependent and increased in cells grown with ethanol. The N-terminal amino acid sequence of the purified enzyme identified the latter as an aldehyde dehydrogenase encoded by *aldB*, which was thought to play a role in the removal of aldehydes and alcohols in cells that were under stress. The purified enzyme showed an estimated molecular mass of 220 ± 8 kDa, consisting of four identical subunits, and preferred to use NADP and acetaldehyde. $MgCl_2$ increased the activity of the NADP-dependent enzyme with various substrates. A comparison of the effect of Mg^{2+} ions on the bacterial enzyme with the effect of Mg^{2+} ions on human liver mitochondrial aldehyde dehydrogenase revealed that the bacterial enzyme shared kinetic properties with the mammalian enzyme. An R197E mutant of the bacterial enzyme appeared to retain very little NADP-dependent activity on acetaldehyde.

Aldehyde dehydrogenases (ALDH) are members of a diverse group of related enzymes catalyzing the oxidation of aldehydes to their corresponding carboxylic acids, with some using NAD and others using NADP as the coenzyme. Their important roles in cellular metabolism have been studied extensively in organisms ranging from bacteria to humans (30). In *Escherichia coli*, more than 10 aldehyde dehydrogenase genes have been identified (26), and one of the genes, *aldB*, mapped from min 80 to 86 on the bacterial chromosome has been predicted to encode an aldehyde dehydrogenase of 512 amino acids (32). Sequence alignments of the deduced amino acid sequence showed 68% similarity and 70% identity with an aldehyde dehydrogenase from *Vibrio cholerae* (22) and an NAD-dependent acetaldehyde dehydrogenase from *Alcaligenes eutrophus* (24), respectively. Expression studies (32, 33) indicated that *aldB* was repressed by Fis (factor for inversion stimulation) but activated by RpoS (RNA polymerase sigma factor) and Crp (cyclic AMP receptor protein), and that the level of expression was highest in the early stationary phase. It was also shown that ethanol induced *aldB* expression. The latter finding, together with the high similarity to the *A. eutrophus* enzyme, suggests that the gene product of *aldB* functions as an acetaldehyde dehydrogenase and that it plays a role in ethanol metabolism through its oxidation of acetaldehyde derived from ethanol. Many free-living, gram-negative bacteria, including *E. coli*, express genes that are crucial to survival at the early stationary phase, and a number of these genes are RpoS dependent (12, 14, 25). The fact that *aldB* is positively regulated by RpoS suggests that it is an important stationary-phase gene (33). With the exception of its gene sequence, little is known about *aldB* at the protein level. The presumed gene product of *aldB* has been classified as an aldehyde dehydrogenase at the EcoCyc website (<http://ecocyc.org>) and is thought

to catalyze some of the reactions ascribed to the gene product of *aldA* (13). Mapped at min 32 on the *E. coli* chromosome, *aldA* has been studied extensively at both the genetic and protein levels (2, 13). The gene product of *aldA*, also known as lactaldehyde dehydrogenase, is an NAD-dependent aldehyde dehydrogenase participating in various metabolic pathways (6, 16, 19). To the best of our knowledge, there is no available literature citation on the catalytic properties of the gene product of *aldB*. Thus, it is difficult to assess the functional relationship between *aldA* and *aldB*.

In a previous study, Heim and Strehler (11) detected a low level of NADP-dependent acetaldehyde dehydrogenase activity in a cell extract of *E. coli* Y1088 while studying an aldehyde dehydrogenase gene, *aldH*. It remains unclear whether this NADP-dependent acetaldehyde dehydrogenase is related to the gene product of *aldH*. We detected a similar NADP-dependent acetaldehyde dehydrogenase first in *E. coli* DH5 α cells transformed with pGEM and later also in nontransformed cells. The NADP-dependent enzyme in the transformed cells has been isolated and characterized. Its N-terminal amino acid sequence, together with the results on growth studies, provides support for it to be the gene product of *aldB*. We have also prepared its R197E variant, which appears to have very little NADP-dependent activity on acetaldehyde. Here, we report our findings and discuss the relationships between the NADP-dependent enzyme and other aldehyde dehydrogenases.

MATERIALS AND METHODS

Bacterial strains and plasmids. DH5 α (pGEM) was originated from an *E. coli* DH5 α clone transformed with a pGEM plasmid. Transformation was conducted with *E. coli* DH5 α competent cells (Life Technologies, Inc.). The pGEM plasmid was prepared by ligation with a pGEM vector and a control DNA insert, both of which originated from a pGEM-T Easy Vector system (Promega). A derivative of pINIII (20), pCJM was used as an expression vector.

Growth conditions. Growth was maintained by shaking partially filled conical flasks in a 37°C incubator set at 200 rpm. Cells were grown in Luria-Bertani (LB) or M9 medium (11) containing 75 μ g of ampicillin/ml.

For the effect of culture age on aldehyde dehydrogenase activity, DH5 α (pGEM) grown overnight in LB was diluted into fresh medium at a ratio of 2:100 and allowed to grow over a period of 10 h. At regular intervals, growth was

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monitored according to the absorbance at 600 nm, and cell samples were harvested. Crude cell extracts prepared from various samples were used for enzyme assays.

For the effect of ethanol on aldehyde dehydrogenase activity, DH5 α (pGEM), grown overnight in LB, was diluted into M9 medium (1:100) and supplemented with 0.1% yeast extract (Difco) and various amounts of ethanol. After 12 h of growth, cells were harvested to prepare crude cell extracts for enzyme assays.

For enzyme purification, batch cultures of DH5 α (pGEM) were grown in 4-liter flasks containing 1 liter of LB. When the optical density at 600 nm reached 2 to 2.5, the cultures were harvested and used for purifying the aldehyde dehydrogenase encoded by *aldB*, henceforth referred to as AldB.

Preparation of crude cell extracts. Cells were harvested by centrifugation at $3,000 \times g$ for 10 min at 4°C. The cell pellets were washed once in 0.9% sodium chloride solution and resuspended in 10 mM sodium phosphate or Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 1 mM dithiothreitol. The cell suspensions were kept at -70°C until they were ready to use. After thawing, the cell suspensions were disrupted by a French Press cell (three cycles at 15,000 lb/in²) prechilled on ice. The insoluble fractions were removed by centrifugation at $30,000 \times g$ for 30 min at 4°C. The remaining soluble fractions were filtered through a 0.8/0.2- μ m-thickness Acrodisc Supor membrane (Gelman) and used as the crude cell extracts.

Purification of AldB. All steps were conducted at 4°C. The crude cell extract, prepared from 40 g of wet cell paste in Tris-HCl buffer, was loaded onto a DEAE-Sephacel column (2 by 22 cm) preequilibrated with the same buffer. After washing with 50 ml of Tris-HCl buffer, the column was eluted with a 500-ml linear gradient of 0.1 to 0.5 M NaCl in the same buffer. Fractions containing a majority of the activity were pooled and dialyzed for several hours against 10 mM sodium phosphate, pH 7.5, containing 1 mM EDTA and 5 mM NaCl. The dialysate was clarified by centrifugation at $30,000 \times g$ for 15 min and loaded onto an affinity column (0.9 by 12.5 cm) of *p*-hydroxylacetophenone (HAP) (10) preequilibrated with sodium phosphate buffer. The AldB-containing fractions were not retained on the column and were collected for ammonium sulfate fractionation. The proteins precipitated at 20 to 60% saturation were resuspended in Tris-HCl buffer and centrifuged as before. The resulting supernatant was loaded onto a Q-Sepharose column (0.8 by 10 cm) preequilibrated with Tris-HCl buffer. The Q-Sepharose column, connected to a fast protein liquid chromatographic system (Amersham Pharmacia Biotech), was eluted with a 400-ml linear gradient of 0 to 20% NaCl, generated by mixing buffer A (Tris-HCl buffer) with buffer B (1 M NaCl in Tris-HCl buffer). Active fractions were pooled, concentrated, and exchanged into 10 mM sodium phosphate, pH 7.5, and 100 mM NaCl using an Amicon cell concentrator fitted with a YM100 membrane. The concentrated sample was loaded onto a 5'AMP-Sepharose column (0.5 by 5 cm) equilibrated with 10 mM sodium phosphate, pH 7.5, containing 100 mM NaCl and 0.5 mM EDTA. The bound enzyme was eluted with a 200-ml linear gradient of 10 to 30 mM sodium phosphate, pH 7.0, containing 100 mM NaCl. Fractions containing homogeneous AldB, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were pooled for subsequent studies.

Enzyme assays. AldB activity was measured at 25°C by determining spectrophotometrically or fluorometrically the reduction of NADP or NAD (9, 28). Measurement of ALDH activity was performed in a 1-ml mixture containing 50 mM sodium phosphate (pH 7.5), 50 to 100 μ l of crude cell extract or 10 to 15 μ g of purified enzyme, 500 μ M NADP or NAD, and various aldehyde concentrations (0.05 to 1 mM). Malate dehydrogenase activity was measured in the same crude cell extracts used for studying the effect of culture age on AldB activity. Measurement of malate dehydrogenase activity with 1 mM oxaloacetic acid and 0.1 mM NADH was performed by a method of Park et al. (21).

Electrophoresis. SDS-PAGE was performed on 10% (wt/vol) slab gels with a discontinuous buffer system (15) in a Bio-Rad Mini-PROTEAN II electrophoresis cell (Bio-Rad). Protein bands were stained with Coomassie brilliant blue (3) and recorded by a photo imaging system (Chemilmager 5500; Alpha Innotech Corporation).

Molecular mass determination. Three methods were used to determine the molecular mass of the purified AldB. First, SDS-PAGE was conducted as described above. The marker proteins (Low Range Control, catalog no. 161-0305; Bio-Rad Laboratories) included phosphorylase B (103 kDa), bovine serum albumin (77 kDa), ovalbumin (50 kDa), carbonic anhydrase (34.3 kDa), and soybean trypsin inhibitor (28.8 kDa). Second, gel filtration chromatography was conducted on a Bio-Rad Bio-Sil SEC250-5 column (300 by 7.8 mm) by using an SP 8800/8810 LC pump (Spectra-Physics). The column was washed with 100 mM sodium phosphate (pH 7.0) and 150 mM NaCl and 20- μ l samples containing ~5 μ g of the purified protein were chromatographed at a flow rate of 1 ml/min. The effluent was monitored by following the absorbance at 280 nm. The column was

calibrated by marker proteins, including ferritin (443 kDa), β -amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), human class 3 aldehyde dehydrogenase (92.5 kDa), bovine serum albumin (66.2 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). Third, mass spectrometry was conducted with a Voyager-DE PRO matrix-assisted laser desorption ionization time of flight instrument (PerSeptive Biosystem Inc.). Pinapinic acid was used as a matrix, and measurement was conducted in a linear mode combined with delayed extension. The scale of the molecular mass was calibrated with bovine serum albumin.

N-terminal sequence and amino acid composition analysis. For amino acid sequencing, a purified sample of AldB was blotted onto a polyvinylidene difluoride membrane (Bio-Rad) after SDS-PAGE (29). Analysis was carried out by an automated protein sequencer (Applied Biosystems Model 477A) in accordance with the manufacturer's instructions. The BLAST program (1), provided by the NCBI website (<http://www.ncbi.nlm.nih.gov>), was used to search for proteins with sequence similarity. Amino acid composition analysis was performed after hydrolysis of proteins with distilled 6 M HCl in evacuated sealed tubes at 150°C for 24, 48, and 96 h. The residues were determined using a Durrum D-500 amino acid analyzer according to the manufacturer's instructions.

Cloning of AldB, oligonucleotide-directed mutagenesis, and gene expression. *AldB* was amplified by PCR using genomic DNA from *E. coli* ATCC 700926D (American Type Culture Collection). Primers corresponding to the N terminus and C terminus of AldB were designed according to the published gene sequence (4). The forward (F) and reverse (R) primers, 5'-GAATTCATATGACCAAT AATCCCCCTTCAGCACAGATTAAGC-3' and 5'-CGGATCCTCAGAACA GCCCAACGGTTTATCCGAGTAGC-3', contained an NdeI and a BamHI site, respectively. PCR was conducted at an annealing temperature of 57°C. A 15-kbp PCR fragment containing *AldB* was cloned into a pGEM-T easy vector (Promega). *AldB* in the recombinant plasmid, pGEMaldB, was amplified by PCR using the F and R primers, digested with NdeI and BamHI, and then cloned into pCJM. To construct the gene for the mutated protein AldB R187E, two other primers, 5'-GTGGTGCTGAAACCTGCAGAGCTTACTCCGCTTTCT GTAC-3' and 5'-GTACAGAAAGCGGAGTAAGCTCTGCAGGTTTCAGCA CCAC-3', were used. These primers were used for PCR with pGEMaldB based on the instructions of a QuikChange XL site-directed mutagenesis kit (Stratagene). The altered *AldB* was then amplified using the F and R primers, digested with NdeI and BamHI, and cloned into pCJM. Proteins were expressed in *E. coli* DH5 α cells by growing the transformed cells at 37°C and inducing them with isopropyl- β -D-thiogalactopyranoside overnight at 16°C when the optical density at 600 nm of the culture reached 0.4 to 0.5.

RESULTS

Crude cell extracts corresponding to different culture ages were measured for aldehyde dehydrogenase activities on acetaldehyde and propionaldehyde with NADP. Malate dehydrogenase activity was measured for the purpose of comparison. The results (Fig. 1) indicate that malate dehydrogenase activity increased initially to a maximum at 4 to 6 h after subculturing and then began to decline. In contrast, aldehyde dehydrogenase activities on acetaldehyde or propionaldehyde initially declined to a minimum at 2 h after subculturing and then increased to a maximum 8 to 9 h after subculturing, during the stationary phase. Among the different cell cultures, the level of enzyme activity with acetaldehyde was consistently higher than that with propionaldehyde. The change in aldehyde dehydrogenase activity on either substrate followed a pattern similar to that of *aldB* expression during the growth course from early log phase to stationary phase (32), suggesting that the gene product of *aldB* is responsible for the enzyme activity. Our second finding, that addition of 0.5 to 2% ethanol increased aldehyde dehydrogenase activity in the crude extracts by twofold, agrees with this suggestion since ethanol has been reported to induce *aldB* expression (32). In the latter study, addition of 2.1% ethanol in the growth medium resulted in about a twofold increase in AldB-LacZ protein fusion activity. It should be noted that an ethanol concentration above 0.25% appeared to slow bacterial growth initially since cultures exposed to ethanol were

FIG. 1. Effect of culture age on malate dehydrogenase (10^{-1}) (▽) and aldehyde dehydrogenase (acetaldehyde, △; propionaldehyde, ○) activities in crude cell extracts. Samples were removed at 1- or 2-h intervals for measuring cell densities (■) and enzyme activities. DH5α(pGEM) was grown as described in Materials and Methods. All values represent the means of three measurements, and similar results were obtained in a duplicate experiment. OD, optical density.

found to take longer than the control culture to reach the stationary phase (data not shown). In order to ensure that the cell cultures at different ethanol concentrations reached the stationary phase before cell harvesting, a predetermined growth period of 12 h was applied to all cultures. Heim and Strehler (11) have conducted a similar study, growing *E. coli* Y1088 cells on 0.3% ethanol for 18 h, and found an increase of NADP-dependent acetaldehyde dehydrogenase activity in the crude cell extract. These authors also reported NAD-dependent acetaldehyde dehydrogenase activity in the extract. However, this NAD-dependent activity was either too low for detection or absent in our extracts.

The scheme for purifying AldB is summarized in Table 1. It involved a number of steps, including ammonium sulfate precipitation and chromatographic separation on different gel matrices. HAP affinity chromatography, originally designed to bind NAD-dependent dehydrogenases (10), was found to be important. Omission of this step resulted in a partially purified AldB with two contaminating proteins as determined by SDS-PAGE, and numerous attempts to remove them by other methods proved unsuccessful. AldB failed to bind to the HAP affinity column, and this tends to support our latter finding, that AldB has a preference for NADP. The homogeneity of AldB was verified by both SDS-PAGE and N-terminal amino acid sequencing. The first 10 amino acid residues from the N terminus were determined to be MTNNPPSAQI. This unique sequence was identical to that inferred from the nucleotide sequence of *aldB* (32). The amino acid composition of AldB was also similar to that inferred from the deduced amino acid sequence of the *aldB* (data not shown). Throughout the purification, the AldB activities with acetaldehyde and propionaldehyde maintained a ratio of about 1.2:1.7 in favor of acetaldehyde. This near-constant relationship implies that both activities were catalyzed by the same enzyme. An apparent mass of about 58 kDa was determined for AldB by SDS-

PAGE. A more accurate molecular mass of 56,352 Da was determined by mass spectrometry. This value is close to the one calculated from the deduced amino acid sequence of AldB (56,306 Da) (32). Under nondenatured conditions, the apparent size of AldB was about 220 ± 8 kDa as determined by gel filtration chromatography on a calibrated column. The elution time (8.2 min) was not affected by protein concentrations ranging from 0.25 to 2.5 $\mu\text{g}/\mu\text{l}$. All these data are consistent with the presence in solution of a stable tetrameric AldB with presumed identical subunits.

The substrate specificity of AldB was investigated at an optimum pH of 7.0, using different aldehydes and coenzymes (Table 2). AldB oxidized a number of tested aldehydes with NADP. Their relative activities increase in the following order: chloroacetaldehyde > acetaldehyde > propionaldehyde > benzaldehyde > mafosfamide > 4-hydroperoxycyclophosphamide. Other tested aldehydes, including glycolaldehyde and glyceraldehyde, were oxidized not at all or insignificantly by AldB with NADP. None of the tested aldehydes were sufficiently oxidized by the enzyme in the presence of NAD. For instance, with 1 to 2 mM NAD and 250 μM acetaldehyde or

TABLE 1. Purification of AldB from DH5α(pGEM-T) cells

Purification step	Vol (ml)	Protein (mg)	Total activity (U) ^a	Sp act (U/mg)	Yield (%)
Cell extract (after filtration)	60	11,250	175	0.015	100
DEAE-Sephacel	52	350	75	0.2	42
HAP affinity column	24	210	70	0.33	40
20–60% (NH ₄) ₂ SO ₄ pellet	30	89	49	0.55	28
Q-Sepharose	35	41	37	0.9	21
5' AMP-Sepharose	4	20	25	2.0	14

^a One unit is defined as the amount of enzyme required to produce 1 μmol of NADPH per minute.

TABLE 2. Activities of AldB relative to activities of acetaldehyde in the presence and absence of 0.4 mM MgCl₂^a

Substrate	Relative activity (%)	
	−MgCl ₂	+MgCl ₂
Acetaldehyde	100	100
Chloroacetaldehyde	193	100
Propionaldehyde	65	57
Glyceraldehyde	1	0
Glycolaldehyde	0	0
Benzaldehyde	40	37
Mafosfamide	21	16
4-Hydroperoxycyclophosphamide	12	20

^a All values represent means of results from duplicate experiments.

propionaldehyde, the enzyme activity obtained was only about 1% of that obtained with the same NADP and substrate concentrations. When assayed with 0.25 to 0.5 mM MgCl₂, NADP-dependent AldB activity on acetaldehyde, propionaldehyde, or benzaldehyde increased by about 100% (Table 2). In the case of chloroacetaldehyde, the enzyme activity remained the same regardless of the presence or absence of MgCl₂. These results are very different from those reported for the substrate specificity of lactaldehyde dehydrogenase (2). To further characterize the substrate specificity of AldB, its kinetic properties with different substrates were determined by bisubstrate kinetics analysis. Several concentrations of NADP (0.05 to 0.5 mM) and aldehyde (0.1 to 1 mM) were used for analysis of the kinetic data. The kinetics constants obtained by a graphic procedure (7) are summarized in Table 3. The results indicated that chloroacetaldehyde and acetaldehyde were preferable substrates for AldB and that acetaldehyde appeared to have a lower K_m , 2.5 μ M. The k_{cat}/K_m values also indicated that the catalytic efficiencies for the two substrates were similar. Another bacterial enzyme reported to have a low K_m for acetaldehyde is *A. eutrophus* NAD-dependent acetaldehyde dehydrogenase (K_m = 4 μ M) (24). Thus, it appears that the two enzymes share not only a high degree of sequence similarity but also a preference for acetaldehyde.

The effect of MgCl₂ on the kinetic properties of AldB was also studied with acetaldehyde. The results (Table 4) indicated that the K_m of AldB for acetaldehyde was not affected by MgCl₂, since the K_m for the substrate varied very little in the presence or absence of MgCl₂. In contrast, the results showed a twofold increase of the dissociation constant (K_{ia}) for NADP in the presence of MgCl₂, suggesting a decreased affinity of the enzyme for the coenzyme.

Sequence alignment comparisons (Fig. 2) show that the NAD-dependent aldehyde dehydrogenases have a glutamate

TABLE 4. Effect of MgCl₂ on the properties of AldB as analyzed by two substrate kinetics^a

Kinetic property (μ M)	Value in the presence of:	
	0 mM MgCl ₂	0.4 mM MgCl ₂
K_m , NADP	65	102
K_m , acetaldehyde	3.5	2.7
K_{ia} , NADP	35	80
V_{max} , acetaldehyde	2	4.5

^a All values represent means of results from duplicate experiments. K_{ia} , dissociation constant.

at a position corresponding to R197 in AldB. To determine the importance of R197 in the coenzyme specificity of AldB, the genes coding for the wild-type enzyme and the R197E mutant were cloned and expressed. Expression of the wild-type enzyme in DH5 α *E. coli* produced a high level of enzyme activity in the crude cell extract. When the R197E mutant was expressed under similar conditions, a low level of enzyme activity was detected in the crude extract, prepared from approximately the same wet cell mass as the wild-type enzyme. When expressed in terms of per-unit protein, the R197E extract had less than 10% of the NADP-dependent acetaldehyde dehydrogenase activity found in the wild-type enzyme's extract. There was no detectable NAD-dependent activity in either cell extract. The AldB activity from individual extracts was further purified by a DEAE-Sephacel step described for the native enzyme from DH5 α (pGEM). The amount of AldB used in individual purifications was adjusted to contain the same enzyme activity. The elution profiles for both enzymes were essentially the same, each being characterized by the presence of an activity peak and an overlapping protein peak. The fractions containing the majority of the activity were then pooled from individual preparations and assayed for enzyme activity and protein concentration. Based on the amount per unit of protein, the NADP-dependent acetaldehyde dehydrogenase activity of the R197E preparation was only a few percent of that found with the wild-type AldB preparation. Again, there was no detectable NAD-dependent activity in either preparation. SDS-PAGE revealed no significant differences between the two preparations in their protein profiles when the same amount of protein from each was electrophoresed. Each had a dominant 58-kDa band with a subunit mass identical to that of the native enzyme from DH5 α (pGEM). The wild-type AldB was purified to homogeneity by the method established for the native enzyme from DH5 α (pGEM), and its specific activity was comparable to that of the purified preparation made from DH5 α (pGEM). Due to the low level of enzyme activity recovered after the DEAE-Sephacel step, we did not further purify the mutant enzyme.

DISCUSSION

Our results clearly indicate that AldB is different from lactaldehyde dehydrogenase in both coenzyme and substrate specificities (2). While AldB is NADP dependent, lactaldehyde dehydrogenase is NAD dependent. Lactaldehyde dehydrogenase is also characterized by high activity on either glycolaldehyde or glyceraldehyde. Under our assay conditions, AldB was unable to oxidize either substrate. Instead, it showed a high

TABLE 3. Kinetic properties of AldB^a

Substrate	V_{max} (U/mg)	K_m (μ M)	K_{cat}/K_m (mM ^{−1} · min ^{−1})
Acetaldehyde	2	2.5	225
Chloroacetaldehyde	3.3	3.6	206
Propionaldehyde	1	5.8	39
Benzaldehyde	0.6	56.8	2
4-Hydroperoxycyclophosphamide	0.2	900	0.05

^a All values represent means of results from duplicate experiments.

FIG. 4. A comparison of the region from H156 to E210 of HALD2 (human liver mitochondria, aldehyde dehydrogenase, NP-000681) with the region from H158 to D212 of the 3D-structure model derived for AldB (*E. coli*). The overlapping structures show that K194 and R197 from AldB are located at positions resembling those of K192 and E195 from HALD2.

on acetaldehyde without Mg^{2+} ions was more than half of that with Mg^{2+} ions. Such findings show that AldB does not behave like the yeast enzyme.

Most of the NAD-dependent aldehyde dehydrogenases studied have two conserved residues, lysine and glutamate, lying close to the adenosine ribose (23). The lysine residue, also conserved in NADP-dependent aldehyde dehydrogenases, interacts with the adenosine ribose in NAD and the 2' phosphate in NADP. The glutamate residue helps coordinate the adenine ribose 2' hydroxyl of NAD and is thought to be responsible for the exclusion of NADP. The alignment of the amino acid sequences of AldB and other NAD-dependent aldehyde dehydrogenases reveals that in AldB, lysine (K194) and arginine (R197) correspond to the conserved residues lysine and glutamate, respectively (Fig. 2). Using the Swiss-Model software at the ExPASy website (<http://swissmodel.expasy.org/>), a three-dimensional (3D) model of the region extending from H158 to D212 of AldB has been constructed using bovine mitochondrial aldehyde dehydrogenase (27) as a template. This region showed a significant 60% sequence identity when compared with the selected region from H156 to E210 of the template. A comparison of their 3D structures (Fig. 4) confirms that K194 and R197 of AldB are located at positions corresponding to those of the conserved K192 and E195 in the bovine mitochondrial aldehyde dehydrogenase, respectively. The arginine residue at position 197 was mutated to a glutamate to learn whether this residue was responsible for the enzyme being an NADP-dependent dehydrogenase. Though the mutant was not purified to homogeneity, it was found to

possess approximately 10% of the catalytic activity of the wild-type enzyme when assayed with NADP and acetaldehyde. Unexpectedly, it did not exhibit any detectable NAD-dependent activity. This residue, while interacting with the coenzyme, is important for overall activity, but does not seem to be the sole determinant for coenzyme specificity. Others have found that they can change the coenzyme specificity of different aldehyde dehydrogenases by making point mutations. Replacement of T175 by a negatively charged amino acid resulted in a change of coenzyme specificity from NAD to NADP and an increase of catalytic efficiency for NAD in *Vibrio harveyi* fatty aldehyde dehydrogenase (34). Another example of changing NAD/NADP specificity preference as a result of replacing just one amino acid (E140) is found in class-3 rat aldehyde dehydrogenase (23). For the respective enzymes, T175 and E140 correspond to R197 of AldB based upon the sequence alignment. Thus, it was surprising to find that the coenzyme specificity did not change in the R197E mutant. A change in the enzyme's catalytic properties can be affected when changing a residue that is in contact with the coenzyme. Mutation of the conserved lysine at position 192 of the human class-2 aldehyde dehydrogenase caused the rate-limiting step of the enzyme to change from deacylation, k_7 , to hydride transfer, k_5 (Fig. 3) (18). Thus, a small alteration in coenzyme binding may have been the cause of the R197E mutant of AldB having so little activity compared to the native enzyme.

Both mammals and *E. coli* possess many different isozymes of aldehyde dehydrogenase. Though some share little identity in sequence or properties, the human mitochondrial enzyme

and AldB have much in common. Most significant is the Mg^{2+} ion effect. Unfortunately, structural data of the human enzyme (17, 27), with respect to the NAD binding domain, cannot be used to explain why AldB uses NADP instead of NAD.

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