Molecular Cloning of the Mitochondrial Aldehyde Dehydrogenase Gene of *Saccharomyces cerevisiae* by Genetic Complementation†

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Mutants of *Saccharomyces cerevisiae* deficient in mitochondrial aldehyde dehydrogenase (ALDH) activity were isolated by chemical mutagenesis with ethyl methanesulfonate. The mutants were selected by their inability to grow on ethanol as the sole carbon source. The ALDH mutants were distinguished from alcohol dehydrogenase mutants by an aldehyde indicator plate test and by immunoscreening. The ALDH gene was isolated from a yeast genomic DNA library on a 5.7-kb insert of a recombinant DNA plasmid by functional complementation of the *aldh* mutation in *S. cerevisiae*. An open reading frame which specifies 533 codons was found within the 2.0-kb *Bam*HI-*Bst*EII fragment in the 5.7-kb genomic insert which can encode a protein with a molecular weight of 58,630. The N-terminal portion of the protein contains many positively charged residues which may serve as a signal sequence that targets the protein to the mitochondria. The amino acid sequence of the proposed mature yeast enzyme shows 30% identity to each of the known ALDH sequences from eukaryotes or prokaryotes. The amino acid residues corresponding to mammalian cysteine 302 and glutamates 268 and 487, implicated to be involved at the active site, were conserved. *S. cerevisiae* ALDH was found to be localized in the mitochondria as a tetrameric enzyme. Thus, that organelle is responsible for acetaldehyde oxidation, as was found in mammalian liver.

Saccharomyces cerevisiae, unlike higher eukaryotes, can metabolize as well as produce ethanol. During fermentation, the consumption of sugars results in the accumulation of ethanol. When glucose or any other fermentable sugar is absent from the culture medium, *S. cerevisiae* can utilize ethanol as the carbon source aerobically. The metabolism of ethanol is well understood in mammals. In the liver, cytosolic alcohol dehydrogenase (ADH) oxidizes ethanol to acetaldehyde and then mitochondrial aldehyde dehydrogenase (ALDH) oxidizes the intermediate to acetate (14). In *S. cerevisiae*, it appears that a mitochondrial alcohol dehydrogenase (ADH3) is responsible for the initial oxidation (65, 67). Little is known about the role of ALDHs in the next step.

Various groups have purified and characterized yeast ALDH (12, 49, 54). It was reported to be a tetrameric enzyme with a subunit molecular mass of 60 kDa and a low K_m for acetaldehyde. Unlike the liver enzymes, the yeast enzyme is activated by K^+ ions (4, 32). Whereas the mammalian enzymes have been sequenced at the cDNA (16, 19, 29, 34) as well as the protein (24, 33, 60) level, no sequence work has been reported for the yeast enzyme.

One of our interests was to study the subcellular localization of acetaldehyde metabolism in *S. cerevisiae*. In the mammalian liver tissue, this was studied by selectively inhibiting the cytosolic or the mitochondrial isozyme of ALDH (11, 52). An alternative approach would be to introduce the enzyme into a cell deficient in ALDH activity. *S. cerevisiae* would be a suitable model system, as it can metabolize ethanol and could serve as a host for the expression of foreign genes (27, 59) and altered yeast genes. It was necessary for us to construct a yeast strain deficient in ALDH activity into which the precursor or mature form of

mitochondrial ALDH could be reintroduced. The mutant was used to identify the gene encoding the yeast mitochondrial ALDH by complementation.

In this paper we report the isolation of a yeast mutant deficient in ALDH activity and the cloning and sequencing of the yeast mitochondrial ALDH gene, and we show that the enzyme is localized in mitochondria.

MATERIALS AND METHODS

Yeast strains, media, and antibodies. *S. cerevisiae* XK25-1B (*MAT α ura3-52*) was provided by G. B. Kohlhaw (Department of Biochemistry, Purdue University). Yeast strains were grown at 30°C in YPD medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose) or in a defined medium containing 0.67% yeast-nitrogen base supplemented with 20 mg of uracil per liter and required amino acids. The carbon source was 2% glucose or 2% (vol/vol) ethanol. Yeast strains harboring derivatives of plasmid YEp24 were grown in medium lacking uracil. Bacterial strains were grown at 37°C in LB medium (0.5% yeast extract, 1% Bacto-Peptone, 1% NaCl, adjusted to pH 7.0) supplemented with ampicillin for selection when necessary. *Escherichia coli* HB101 (7) was used for the storage and amplification of yeast-*E. coli* shuttle plasmids. *E. coli* JM101 (68) was used for phage infection for the preparation of double-stranded and single-stranded M13 DNA for sequencing. The antiserum against ALDH was raised in rabbits (30) by injecting commercial yeast ALDH. The serum was treated with 40% ammonium sulfate to precipitate the immunoglobulin G fraction. After dialysis against phosphate-buffered saline buffer (10 mM sodium phosphate buffer containing 0.9% sodium chloride, adjusted to pH 7.4), the antiserum was stored at –20°C.

Isolation of mutants. Chemical mutagenesis with ethyl methanesulfonate was performed essentially as described by Fink (17) with some modifications. *S. cerevisiae* was grown in YPD overnight at 30°C. The cells were washed twice with sterile water and suspended in 10 ml of sodium phosphate

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buffer (0.1 M, pH 8.0). Ethyl methanesulfonate (0.3 ml) was added, and the tube was incubated at 30°C for 50 min without shaking. The cells were pelleted, washed three times with sterile water, and incubated for 10 min with sodium thiosulfate (6%) to inactivate ethyl methanesulfonate. This was followed by a 1-h nystatin treatment (20 µg/ml) for mutant enrichment (47). The cells then were washed twice with sterile water and spread on YPD plates after serial dilutions in order to obtain 200 to 300 colonies per plate. The colonies in the master plate were replica plated on minimal medium containing ethanol (2%, vol/vol) as the carbon source. After 2 to 3 days of incubation at 30°C, the colonies that did not grow were picked from the master plate and transferred to a fresh plate for storage and further analysis.

Aldehyde indicator plate test. Indicator plates were prepared by the method of Conway et al. (13). Yeast colonies grown on YPD medium were transferred to indicator plates with sterile wooden applicator sticks and incubated at 30°C in the dark for 6 to 12 h.

Yeast colony hybridization and immunoscreening. Yeast colonies were transferred to nitrocellulose and lysed in the presence of NaOH and 2-mercaptoethanol as described by Lyons and Nelson (39). The presence of ALDH protein was detected with antiserum (1:7,500) raised against commercial yeast ALDH.

Yeast transformation and DNA manipulation. Yeast strains were transformed by utilizing the spheroplast formation procedure (26) or the alkaline cation treatment of intact cells (31). Plasmid DNA from yeast cells was isolated as described by Nasmyth and Reed (43). Transformation of *E. coli*, small scale plasmid DNA preparation from *E. coli*, restriction enzyme digestions, and DNA ligations were accomplished by using standard techniques (40). DNA sequencing was performed by the dideoxy-chain termination method (46).

Preparation of crude homogenate. Yeast cells were suspended in 50 mM sodium phosphate buffer (pH 7.5) (1 g [wet weight] of cells per 2.5 ml of buffer) containing 1 mM EDTA, 2 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride, and 2 mM benzamidine and then lysed in a French pressure cell at 1,100 lb/in². The cell debris was removed by centrifuging the extract at 10,000 × g for 10 min at 4°C.

Isolation of mitochondria. The protoplast formation method of Jacobson and Bernofsky (32) was used with the following changes. The yeast cells were converted to spheroplasts by enzymatic digestion of the cell wall with lyticase (3 mg/g [wet weight] of cells). The mitochondria were released from the protoplasts by low pressure treatment in a French pressure cell (400 lb/in²) and collected by differential centrifugation. The yield of mitochondrial protein was 3 to 4 mg/g (wet weight) of cells. The mitochondria were quickly frozen and stored at -70°C.

Preparation of mitochondrial extract. Mitochondria were resuspended in isolation buffer and disrupted at 4°C by sonication with three 30-s cycles at medium intensity (40% duty cycle) with a Heat Systems model W-225 ultrasonic generator. Disrupted mitochondria were centrifuged at 15,000 rpm for 15 min at 4°C to sediment the membranes.

Protein analysis and enzyme assay. Protein concentration was calculated by using Bio-Rad protein determination reagents, as described in the manufacturer's instruction book, with bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (37), and proteins were transferred to nitrocellulose sheets by the method of Towbin et al. (56) and then probed with antiserum. Continuous

TABLE 1. Subcellular activity of ALDH^a

Enzyme	Activity in:	
	Mitochondria	Cytosol
ALDH		
+K ⁺	31,700	4,000
-K ⁺	5,600	750
Fumarase	11,750	1,970

^a Activity is expressed as nanomoles per minute per 10 g of cells. Data were not corrected for percent recovery of mitochondria. In the presence and absence of 40 mM K⁺ ions, 88% of the ALDH activity was found in the mitochondrial fraction and 12% was found in the cytosolic fraction. Since 14% of the fumarase was found in the cytosolic fraction, it can be concluded that the activity of ALDH in the cytosol was due to mitochondrial contamination.

molecular sieve gradient PAGE was performed by the method of Margolis and Kendrick (41). Isoelectric focusing of the samples was done on agarose gels with Pharmalytes (pH 4 to 6.5) by using an FBE 3000 Pharmacia horizontal electrophoresis apparatus. The activity band was visualized by staining with nitroblue tetrazolium (1 mM) and phenazine methosulfate (1.25 mM) at 37°C in the dark. The reaction mixture contained NAD⁺ (1.5 mM) and acetaldehyde (180 mM). Pyrazole (3.5 mM) and pyruvic acid (6 mM) were added as inhibitors of ADH and lactate dehydrogenase, respectively. The ALDH activity was assayed fluorometrically in 50 mM sodium pyrophosphate buffer (pH 8.3) at 25°C. The reaction mixture contained acetaldehyde (1.8 to 180 µM), bovine serum albumin (0.1%), and enzyme in a total volume of 1 ml. The assay was performed in the presence and absence of potassium chloride (40 mM). Fumarase activity was assayed by the method of Racker (45).

Plasmids and the clone bank. The clone bank used for screening, a YEp24 genomic library (6), was provided by G. B. Kohlhaw. Plasmids pDS1 and pDS2 are YEp24 plasmids containing genomic inserts that complement the growth of the mutant on ethanol minimal medium. M13mp18 and M13mp19 (42) were used in DNA sequence analysis by the dideoxy-chain termination method.

Materials. All restriction enzymes, DNA ligase, and Klenow fragments were obtained from New England BioLabs. Calf alkaline phosphatase and yeast ALDH were from Boehringer Mannheim Biochemicals. The T4 DNA polymerase I was from Amersham. The DNA sequencing kit was obtained from United States Biochemicals. Manufacturers' instructions were followed for all enzymatic reactions. For resolving some GC-rich regions of DNA, a sequencing kit containing *Taq* DNA polymerase I from International Biotechnologies Inc. was used. [α -³⁵S]dATP was from Amersham. Nitrocellulose filters were from Schleicher and Schuell. Lyticase was from Sigma Chemical Co. All other chemicals were of analytical grade.

Nucleotide sequence accession number. The GenBank accession number for the aldehyde dehydrogenase gene is M57887.

RESULTS

ALDH activity in wild-type *S. cerevisiae*. *S. cerevisiae* (XK25-1B) possesses ALDH activity which could be detected by assaying the crude cell homogenate or after isoelectric focusing. Cells grown aerobically on glucose and harvested in the stationary phase were found to have 325 U of activity per g (wet weight) of cells. When the cells were

FIG. 1. Subcellular distribution of ALDH activity as determined by isoelectric focusing gel electrophoresis. Mitochondrial and cytosolic fractions were separated from yeast extract, and the bands were visualized by staining for activity. Lane 1, standard markers; lane 2, yeast crude cell homogenate; lane 3, isolated mitochondrial fraction; lane 4, cytosolic fraction. The bands above pI 6.0 were found to be present in the absence of NAD or acetaldehyde. One activity band was found in the mitochondrial fraction (pI, 5.5 ± 0.1), while no activity band was seen in the cytosolic fraction.

grown aerobically with ethanol as the only source of carbon, the activity was 300 U. This shows that the enzyme was not induced by the alcohol.

Approximately 90% of the enzyme activity was found to

FIG. 2. Identification of ALDH protein in wild-type *S. cerevisiae*. Western blot analysis of the ALDH mutant and the transformant are shown. The protein samples were separated by SDS-PAGE on a 10% gel and transferred to a nitrocellulose membrane. The proteins were detected by antiserum. Lane 1, the yeast transformant containing the 5.7-kb genomic DNA insert; lane 2, transformant containing the 2.5-kb *Bgl*II-*Bgl*II fragment (see Fig. 4); lane 3, transformant containing the 2.0-kb *Bam*HI-*Bam*HI fragment (see Fig. 4); lane 4, the ALDH mutant (DSW127); lane 5, wild type (XK25-1B). A separate experiment showed that the subunit molecular mass was 60 kDa.

FIG. 3. Molecular mass of the mature ALDH protein. The protein samples were separated on a 5 to 15% gradient gel run at 4°C for 24 h. The proteins were transferred electrophoretically to a nitrocellulose membrane. The immobilized proteins were detected by antiserum raised against commercial yeast ALDH. Lane 1, mitochondria isolated from the wild type (XK25-1B); lane 2, ALDH⁻ strain (DSW127); lane 3, the transformant (DS730). Marker proteins (not shown) were used to calibrate the gel. The mature protein had a molecular mass of 240 kDa.

be associated with the mitochondrial fraction (Table 1). Addition of K⁺ ions increased the activity of both the cytosolic and mitochondrial fractions by sixfold. Fumarase activity was used as a marker for mitochondrial contamination of the cytosolic fraction; approximately 10% of the activity was associated with it. Thus, it appears that ALDH is a mitochondrial enzyme and that the small amount of activity found in the cytosolic fraction was due to contamination by lysed mitochondria.

To determine whether there were isozymes of ALDH, isoelectric focusing of the crude homogenate and of the separated cytosolic and mitochondrial fractions was performed. Essentially, one major band of activity was observed after focusing the crude homogenate, as shown in Fig. 1. The band corresponded to a protein with a pI of 5.55 ± 0.1 . An identical band was observed in the mitochondrial fraction. Western immunoblotting after SDS-PAGE showed that ALDH had a subunit molecular mass of ca. 60 kDa (Fig. 2). Gradient gel electrophoresis showed that the enzyme was a tetramer (Fig. 3).

The K_m for acetaldehyde with the crude mitochondrial enzyme was estimated to be 20 μ M at pH 8.3. The presence of K⁺ ions not only increased the V_{max} but decreased the K_m

FIG. 4. Restriction maps of the recombinant plasmids pDS1 and pDS2 carrying the yeast ALDH gene. The thin lines indicate the yeast DNA. The bold lines indicate the overlapping region. The shaded boxes represent vector DNA. The orientation and the extent of overlap between the two cloned yeast DNA segments were determined by electrophoretic analysis on agarose gels of the fragments produced by the restriction digestion of the plasmid with *Bam*HI, *Sal*I, *Kpn*I, *Bst*EII, and *Bgl*II. The estimated sizes of the cloned inserts in pDS1 and pDS2 are approximately 5.7 and 9.0 kb, respectively.

to 5 μ M. The activation by K^+ ions and the K_m values are similar to those previously reported (50).

Isolation and selection of *aldh* mutants. Yeast cells were treated with the chemical mutagen ethyl methanesulfonate. Lack of growth on ethanol was used as a selection criterion for identifying potential *aldh* mutants. From the 3×10^6 colonies screened, 75 mutants were isolated that did not grow on ethanol. The growth of the mutants on glucose was similar to that of the wild type. Thus, we inferred that the mutants were impaired in one of the enzymes required for ethanol metabolism. In order to distinguish *aldh* mutants from *adh* mutants, an aldehyde indicator plate test and colony hybridization with antibodies were performed. Aldehyde indicator plates were used to identify yeast mutants producing acetaldehyde by the action of ADH. The colonies possessing ADH but not ALDH activity appeared intensely red because of the accumulation of acetaldehyde. The colonies which did not possess ADH or which contained both enzyme activities appeared white.

Colony hybridization was used to identify mutants void of ALDH protein. The same 75 mutants that did not grow on ethanol were screened with antibodies. Eleven mutants were found to be void of ALDH and accumulated acetaldehyde, as determined by the aldehyde plate test. Mitochondria were isolated from these mutants and assayed for ADH and ALDH activity. All showed the same level of ADH activity as that of the wild-type strain. The mutant which possessed the lowest ALDH activity (3% that of the wild type), to be called DSW127, was used to isolate the ALDH gene from a yeast genomic DNA library.

Isolation of the ALDH gene. The ALDH gene was identified on a recombinant DNA plasmid by its functional complementation of an *aldh* mutation in DSW127. A recombinant DNA plasmid library constructed in YEp24 was used to transform DSW127 (*MAT α ura3-52*). Plasmids containing the ALDH gene were selected in two steps. In the first step,

ura⁺ transformants were obtained at a frequency of 1,200 transformants per μ g of DNA by growth on medium lacking uracil. The *ura*⁺ transformants were pooled and spread on plates containing ethanol minimal medium without uracil and were screened for *ura*⁺ transformants capable of growth with ethanol as the sole carbon source.

Plasmid DNA isolated from these transformants was used to transform *E. coli* HB101, and the transformant colonies were selected by ampicillin resistance. The transformants were grown in LB medium for large scale plasmid DNA preparations. The plasmid was isolated and subjected to restriction enzyme analysis. A physical map of the plasmid is shown in Fig. 4. The recombinant plasmid that complemented the growth of the mutant on ethanol minimal medium contained an insert of approximately 5.7 kb.

FIG. 5. A partial restriction map of the ALDH gene and the nucleotide sequencing strategy. The arrows indicate the direction and the extent of the nucleotide sequence. The DNA was subcloned in phages M13mp18 and M13mp19, and the sequence was determined in both orientations. The position of the open reading frame is shown by the open box.

FIG. 6. Nucleotide sequence of the ALDH gene and the flanking regions. Nucleotides upstream from the translation start site carry negative numbers, and those downstream carry positive numbers. The deduced amino acid sequence of the open reading frame is shown above the nucleotide sequence. The putative TATA sequence is boxed, and transcription termination and polyadenylation signals are underlined.

In order to obtain other independent recombinant plasmids, the library was rescreened and more *ura⁺ ald^h* yeast transformants were identified. One more independent plasmid was found. It contained a genomic insert of 9.0 kb. The two plasmids were found to have overlapping restriction maps with a common region of 3.3 kb, as shown in Fig. 4. Both plasmids were used for retransformation of DSW127, and all of the *ura⁺* transformants could grow on ethanol, thus showing the presence of the ALDH gene. Mitochondria were isolated from these transformants and were now shown to possess ALDH activity. Western blot analysis verified that the complemented protein was ALDH (Fig. 2 and 3).

Nucleotide sequence of the ALDH gene. The *S. cerevisiae* gene encoding mitochondrial ALDH was localized to a 3.3-kb overlapping *Bgl*II-*Bgl*II restriction fragment of the two independent plasmids, pDS1 and pDS2 (Fig. 4). Various fragments were subcloned in M13mp18 and M13mp19 vectors, and the nucleotide sequence was determined in both orientations. A single long open reading frame was found when the 0.8-kb *Bam*HI-*Sal*I fragment was sequenced. Partial nucleotide sequence analysis extending from the *Bam*HI restriction site near the 5' end of the coding region established the orientation of the coding region, because when the complementary strand was sequenced in the *Sal*I to *Bam*HI direction, no open reading frame was found. This means that transcription of the gene starts at *Bam*HI and proceeds towards *Sal*I. A partial restriction map and the strategy for nucleotide sequence analysis of the ALDH gene are illustrated in Fig. 5. The complete sequence of the gene is shown in Fig. 6. An open reading frame starting with the ATG at position 1 and ending with the stop codon TAA at position 534 could encode a polypeptide with a molecular weight of 58,630, which agrees well with the 58- to 60-kDa band observed on SDS-polyacrylamide gels.

Several regions which are homologous to the consensus sequence TATAA/TAA/T are believed to be part of a eukaryotic promoter element were found (positions -88, -109, -118, -130, and -171) (9, 51). A pyrimidine-rich sequence, CTTTATTCTTC, was found between -162 and -152, implicated to be the mRNA capping site (55). The sequence CAAG, located at position -71, is consistent with the sequence reported to be involved in transcription initiation (10). The nucleotide sequences TAAG and GATAA near the presumed initiation codon conform to the general sequence motifs pyAApu and pupypupu, respectively, found at the major transcription initiation sites in yeast genes (15, 20, 22).

At position -3 from the first ATG in the open reading frame there is an A. However, there is no purine at position +4, as was found in many other sequences (36). Neither rat nor beef ALDH has a purine at position +4 (18). The absence of other AUG codons in the 5'-flanking region supports the conclusion that the methionine at position 1 in Fig. 6 is the first translated amino acid of the mitochondrial ALDH precursor protein. At the 3' end of the ALDH gene, a consensus polyadenylation signal, AATAAA (3), is found at position 1656, which is followed by a consensus tripartite transcription termination signal, TAG. . . TAGT. . . TTT (70), at positions 1669, 1681, and 1695, respectively.

DISCUSSION

Yeast ALDH was first purified by Steinman and Jakoby (49), who found that the enzyme was activated by K^+ ions, and later by others (48, 54). Though some detailed kinetic studies were performed (5, 8, 12, 50), neither the amino acid

FIG. 7. Amphipathic alpha-helical wheel projection of the first 21 N-terminal amino acids presumed to be the signal sequence that targets the protein to the mitochondria. The charged residues are indicated (+).

sequence nor the subcellular localization of the enzyme has been reported. Since no protein sequence information was available, we chose to create a yeast mutant deficient in ALDH activity in order to clone the gene by complementation.

We found that the ALDH activity was primarily localized in the mitochondria. No evidence was found to suggest that a cytosolic isozyme existed. The enzyme had a low K_m for acetaldehyde and was activated by K^+ ions, consistent with the previous reports of Steinman and Jakoby (50). A high K_m for acetaldehyde (0.667 mM) was found by Tamaki et al. (54) when they used Tris-HCl buffer (pH 8.3) and lysed the cells in 50 mM potassium phosphate buffer. We also found that Tris buffer increased the K_m for the substrate (unpublished data).

The ALDH gene was isolated on a recombinant DNA plasmid and was identified by its ability to complement the *aldh* mutation in the DSW127 strain. The ALDH activity in the isolated mitochondria of the transformants was essentially the same as that found in the wild-type *S. cerevisiae*. The facts that the *aldh* mutant did not grow on ethanol and that it accumulated acetaldehyde, while the transformants could grow on ethanol, suggest that the mitochondrion is the compartment for aldehyde oxidation just as in the mammalian liver (11). The finding that ALDH activity was localized in yeast mitochondria proves this.

Two independent plasmids containing genomic inserts were capable of complementing the growth defect of the mutant. It was possible to localize the ALDH gene for

Beef (19)	M L R A V A L A A A R L G P R Q G R R L L S A
Human (29)	- - - - - F - - - - - S A
Rat (16)	- - - - - S T - - - R - - - L S - - - S A
Yeast ^a	- - A T R N F V P I I R A S I K W - I K - S A

FIG. 8. Comparison of the signal sequences of yeast and mammalian mitochondrial ALDHs. ^a This study.

sequencing from the overlapping regions of the two inserts. A unique open reading frame encoding a protein of 533 amino acids was found from DNA sequencing.

It is known that a signal sequence is required for a protein to be imported into mitochondria. All signal sequences contain many positively charged amino acids and no acidic residues (62). An amphipathic alpha-helical wheel can be drawn in which the positively charged residues reside on one side and the hydrophobic residues reside on the opposite side (61). The first 21 amino acids at the N terminus are a potential signal sequence because they contain many basic residues, are void of acidic ones, and can form an amphipathic alpha-helical wheel as shown in Fig. 7. In all mammalian mitochondrial ALDH sequences the mature protein starts with a serine which is followed by an alanine (Fig. 8). Therefore, serine and alanine at positions 22 and 23, respectively, in the yeast sequence are the potential start of the mature protein. Finally, statistical analysis of signal sequences indicates that a lysine or arginine is often found at position -2 from the site where the mitochondrial matrix space protease cleaves the signal sequence (21). The yeast enzyme has a lysine at this position. Thus, it is presumed that the mature protein will contain 512 amino acids. These residues were compared and aligned with the already known sequences of ALDHs from the mammalian liver, *Aspergillus nidulans*, *Pseudomonas oleovorans*, and spinach, as shown in Fig. 9.

Mammalian ALDHs can be divided into three classes on the basis of their primary structures. Class 1 ALDHs are the cytosolic enzymes, class 2 ALDHs are the mitochondrial enzymes, and class 3 ALDHs include tumor-specific, microsomal, and stomach enzymes. There is approximately 70% identity between each class. Yeast ALDH was found to possess the same percentage (ca. 30%) of identity to each class of mammalian enzymes (28, 34, 60). It was unexpected to find the same degree of identity between the yeast mitochondrial enzyme and the *Pseudomonas* (35), *Aspergillus* (44), and spinach (66) enzymes. Even when homologies were compared, the yeast enzyme was as similar to the mammalian enzymes as it was to the others.

Though the overall identity between the yeast enzyme and the other enzymes was 29%, a higher identity was found in the C-terminal half of the proteins. There was 40% identity in the 284 amino acid residues starting from amino acid 180, with homology increasing to approximately 60%. Even among the mammalian enzymes greater identity exists in the C-terminal half of the molecule. Though the coenzyme-binding domain has not been identified, it has been postulated to exist in the C-terminal portion of the enzyme (23).

The active site of ALDH is not known. It has been proposed that the oxidation of the aldehyde occurs through covalent catalysis. Some residues postulated to exist at the active site include a nucleophile and a possible general acid or base (53). Though the nucleophilic residue has not been identified with certainty, strong evidence exists suggesting that it is a cysteine residue (23). We proposed that it could be at position 49 or 162 (57, 58). By performing site-directed mutagenesis with the rat liver enzyme, we now find that the residue is cysteine 302 (63), as originally suggested by Hempel and Pietruszko (25). Recently, however, it was found that in sheep liver cytosolic ALDH, serine 74 could be modified by a substrate (38). *S. cerevisiae* does not possess serine 74 nor cysteines 49 and 162 but does have cysteine 302. Thus, this residue remains the best candidate for the active site nucleophile, for it is conserved in all the known ALDH sequences.

It was reported that glutamate 268 in the human cytosolic enzyme appeared to be essential for activity (1, 2). This is also a highly conserved residue in all species. Glutamate 487, found to be essential for human enzyme activity (69), is present in *S. cerevisiae* but not in all others. A histidine has been proposed to be involved in catalysis (64). The histidine at position 235 is highly conserved but is not found in the *Pseudomonas* or tumor enzyme. Thus, though yeast ALDH shows overall sequence homology with all other ALDHs tested, it appears to be more similar to the mammalian class 1 and 2 enzymes than to the others. It should be possible, then, to use yeast ALDH to verify the essentiality of various amino acid residues determined by using chemical modification and site-directed mutagenesis.

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