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Mitochondrial NAD Dependent Aldehyde Dehydrogenase either from Yeast or Human Replaces Yeast Cytoplasmic NADP Dependent Aldehyde Dehydrogenase for the Aerobic Growth of Yeast on Ethanol

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Abstract

Background—In a previous study, we deleted three aldehyde dehydrogenase (ALDH) genes, involved in ethanol metabolism, from yeast *S. cerevisiae* and found that the triple deleted yeast strain did not grow on ethanol as sole carbon source. The ALDHs were NADP dependent cytosolic ALDH1, NAD dependent mitochondrial ALDH2 and NAD/NADP dependent mitochondrial ALDH5. Double deleted strain ΔALDH2+ΔALDH5 or ΔALDH1+ΔALDH5 could grow on ethanol. However, the double deleted strain ΔALDH1+ΔALDH2 did not grow in ethanol.

Methods—Triple deleted yeast strain was used. Mitochondrial NAD dependent ALDH from yeast or human was placed in yeast cytosol.

Results—In the present study we found that a mutant form of cytoplasmic ALDH1 with very low activity barely supported the growth of the triple deleted strain (ΔALDH1+ΔALDH2+ΔALDH5) on ethanol. Finding the importance of NADP dependent ALDH1 on the growth of the strain on ethanol we examined if NAD dependent mitochondrial ALDH2 either from yeast or human would be able to support the growth of the triple deleted strain on ethanol if the mitochondrial form was placed in cytosol. We found that the NAD dependent mitochondrial ALDH2 from yeast or human was active in cytosol and supported the growth of the triple deleted strain on ethanol.

Conclusion—This study showed that coenzyme preference of ALDH is not critical in cytosol of yeast for the growth on ethanol.

Keywords

Aldehyde dehydrogenase; co-enzyme specificity; mitochondria; aerobic growth

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This manuscript is dedicated to Dr. Henry Weiner.

1. Introduction

It is not always apparent why an enzyme is found any one subcellular compartment. In some cases, such as for the enzymes involved in the TCA pathway, it is apparent that they need to be located near each other in mitochondria so the NADH can readily enter the electron transport system and not rely upon a shuttle system to bring the electrons from NADH into the organelle. In contrast, the two step metabolism of ethanol to acetate takes place in two subcellular organelles in mammalian liver [1,2]. The first step is catalyzed by an NAD-dependent alcohol dehydrogenase (ADH) located in the cytosol while the second step is catalyzed by an NAD-dependent ALDH located in the matrix space of the mitochondria even though isozymes of the ALDH are found in cytosol [3]. It might be argued that the enzymes involved in the conversion of ethanol to acetate are in separate compartments so there would not be as large demand on the subcellular NAD pool as would occur if both oxidation steps took place in the cytosol. When the concentration of ethanol is high, lactate is produced suggesting that the cell is trying to reestablish the NAD/NADH ratio and implying that the shuttle systems cannot bring the reducing equivalents fast enough into the mitochondria. In an analogous way, fermenting yeast only produces ethanol to regenerate the NAD pool so the cytosolic reactions involved with glucose metabolism can proceed at a fast rate. In yeast, the enzymes involved in the oxidation of ethanol are found in different subcellular organelles [4,5]. The first step of the oxidation is carried out by cytosolic ADH [6,7,8,9] while the next step from aldehyde to acetate is carried out by either mitochondrial ALDH or cytosolic ALDH [5,10,11,12]. The cytosolic ALDH is encoded by the *ALD6* [9,10], while the mitochondrial isozyme are encoded by *ALD4* and *ALD5* [8,11,12]. With these enzymes, though, the coenzyme requirements are different. The cytosolic ALDH is an NADP-dependent enzyme while ADH and mitochondrial ALDH are NAD-preferring enzyme [13,14,15]. Ethanol, though at high concentrations affect membranes and causes intoxication to mammals, is not as cytotoxic as is acetaldehyde. Since the latter is primarily oxidized in the mitochondria after being formed in the cytosol, it is not apparent what were the evolutionary pressures that caused the compound to have to diffuse from cytosol into mitochondria prior to being oxidized to acetate. Acetate can freely diffuse into mitochondria so its conversion to acetyl CoA should not be a reason for the compartmentalization though the formation of the acetyl CoA occurs in mitochondria.

Using a yeast strain developed in our laboratory that was lacking all ALDHs involved in acetaldehyde oxidation, we were able to show that during the aerobic growth with ethanol as the sole carbon source, both the mitochondrial and the cytosolic isozymes could support the growth to the same extent when expressed separately [5]. This finding questions the importance of subcellular localization of ALDHs. Using selective inhibitors, though, we showed that rat liver cytosolic isozyme really did not contribute to the oxidation of acetaldehyde during the metabolism of ethanol in liver slices [2]. It is known that humans lacking an active mitochondrial ALDH cannot metabolize acetaldehyde well even though they possessed an active cytosolic isozyme of the enzyme.

In this study, the NAD dependent yeast or human mitochondrial ALDH2 was expressed in the cytosol of the triple deleted yeast strain to test if it can complement the NADP dependent cytosolic ALDH1. This study also examines if coenzyme preference is a determining factor for the growth of the deleted strain on ethanol as sole carbon source.

2. Materials and Methods

2.1. Strain, Media and Growth Conditions

The wild type *S.cerevisiae* strain used was TWY 973(*Mata, ura3, his7, trp1, leu2*) from Prof. Weinert's laboratory in the University of Arizona. The triple disruption strain

(ΔyALDH1 (cytosolic/ΔyALDH2(mitochondrial)/ΔyALDH5(mitochondrial) was employed from our previous study [5]. Yeast medium used in this study was synthetic minimal medium (SM) with either 2% glucose (called SC) or 3% ethanol as the carbon source. It consisted of 0.67% yeast nitrogen base (Difco), 2–3% required carbon source, and amino acid omission mixtures (BIO 101, Inc), deficient in the appropriate amino acids required for selection. Synthetic medium supplemented with ethanol was supplemented with 0.05% glucose to facilitate growth of disrupted strain. Cells were cultured in liquid culture or 2% agar plates at 30°C. Yeast transformations were conducted using the lithium acetate protocol [5]. *Escherichia coli* DH5*α* strain was used for cloning work.

2.2. Miscellaneous

Procedures for DNA purification, restriction enzyme digestion, ligation, and *E. coli* transformations were performed using standard protocols. PCRs were carried out with Vent DNA polymerase (New England Biolabs). All oligonucleotides were synthesized by Integrated DNA Technology. DNA sequencing was performed by Purdue University Sequencing Center. *S.cerevisiae* chromosomal DNA was prepared according to standard method.

2.3. Cloning of yeast cytosolic *ALDH* (yALDH1) and mitochondrial *ALDH* (yALDH2) genes in pET24a

Based on the published sequence of cDNA of yeast cytosolic ALDH1, primers were designed to obtain the 1.5kb fragment of cytosolic ALDH1 from yeast genomic DNA using PCR. The fragment was digested with *Nde1* and *Xho1* and ligated to *Nde1* and *Xho1* cut pET24a vector from Novagen. pET24a is designed to have six histidine residues fused to the C-terminus of the interested protein to facilitate its purification. Single and double mutations were performed by PCR. Yeast mitochondrial ALDH2 was also cloned in pET24a similarly as done for cytosolic ALDH1. Bl21 cells (Novagen) were transformed with the plasmids for expression in bacteria.

2.4. Construction of yeast Expression Vector

Based on the yeast cytosolic *ALDH* (*yALDH1*) sequence two primers were designed to amplify the 524 bp fragment containing the ALDH1 promoter region, which was located upstream of yeast ALDH1 coding region. PCR was performed using *S. cerevisiae* chromosomal DNA as the template. The PCR product was digested with *EcoR I* and *Nde I* and cloned into *EcoR I /Nde I* site of plasmid pYXCJM, a derivative of pYX112, kindly provided by Prof. Charbonneau (Purdue University), in which the original *Nde I* was removed from the plasmid backbone and the new *Nde I* was introduced in the multiple cloning site by our laboratory. This intermediate plasmid was called pYX/yaldh1-NP. Plasmid pT7-7/yALDH1 carrying the yeast cytosolic ALDH1 coding regions was cut with *Nde I* and *BamH I*, and a 1.5 kb fragment was inserted into *Nde I* and *BamH I* site of the pYX/yaldh1-NP. The plasmid was called pYX/yaldh1-NP/ yALDH1. Then, two primers were designed to amplify the 704-bp fragment of the yeast ALDH terminator region, which was the sequence of the downstream of yeast ALDH1 coding region. PCR was performed using the *S. cerevisiae* chromosomal DNA as template. The PCR product was digested with *BamH I* and *Xhol I* and subcloned into the *BamH I /Xhol* site of the plasmid pYX/yaldh1-NP/yALDH1. The new plasmid was called pYX/yaldh1-NP-TM/yALDH1. Similarly, the plasmid termed pYX/yaldh2-NP-TM/yALDH2 was constructed by following the above procedures for mitochondrial ALDH2.

To construct a plasmid for expression of ALDH, a single copy pRS 316 plasmid, kindly provided by Prof. Gunter Kohlhaw (Purdue University), was modified by removing the original *Nde I* in the plasmid backbone. The modified plasmid was named pRS316M. The

plasmid pYX/yaldh1-NP-TM/yALDH1 was digested with *Not I* and *Xho I* and the 2.74 kb fragment, including yeast ALDH1 promoter, coding and terminator region, was subcloned into *Not I/Xho I* site of pRS316M. This plasmid for expression of cytosolic ALDH1 was named as pRS316M/yALDH1. The plasmid pYX/yaldh2-NP-TM/yALDH2 was cut by *EcoR I* and *Sal I* and the 2.28 kb fragment, containing yeast ALDH2 promoter, coding and terminator region, was inserted into EcoR I/Sal I of plasmid pRS316M. The plasmid for expression of yeast ALDH2 was termed as pRS316M/yALDH2. The correct orientation of yeast ALDH1 and yeast ALDH2 promoter and terminator fragment were confirmed by sequencing. To construct the plasmid used for the expression of human ALDH, pT7-7/hALDH1 (cytosolic) and pT7-7/hALDH2 (mitochondrial) were digested with *Nde I* and *BamH I* and the 1.5 kb fragments were inserted into *Nde I /BamH I* site of pRS316M/yALDH1and RS316M/yALDH2, respectively. These constructions were called pRS316M/hALDH1and RS316M/hALDH2, respectively.

The cDNA encoding the liver mitochondrial precursor pALDH2 was cut from plasmid pT7-7/hpALDH2 and then cloned in to plasmid pRS316M/hALDH2m between *Nde I* and *BamH I*. The resulting vector was called pRS316M/hpALDH2. To make the human ALDH1 chimeric precursor, the human liver mitochondrial ALDH2 leader was amplified by PCR using the pT7-7/hpALDH2 as a template. The PCR product was purified and then used for next PCR reaction as a primer. The second PCR was performed by using above primer and human ALDH1 C-terminal primer, and the pT7-7/hALDH1 as template. The PCR product was digested with *Nde I* and *Bam H I* and ligated into the *Nde I /Bam HI* of pRS316M/yALDH2. This construct was confirmed by DNA sequencing and named as pRS316M/hpALDH1. The yeast ALDH1 chimeric precursor was made by the same procedure using pT7-7/ypALDH2 as a template. The first PCR and C-terminal primer of yeast ALDH1 that included a *BamH I* site were used to make the yeast ALDH1 precursor. The PCR product was digested with Nde1 and *BamH1* and ligated into the *Nde I/BamH I* of pRS316/yALDH1. The resulting construction was sequenced and called pRS316M/ypALDH1. To clone the mature portion of yALDH2 in expression vector, a PCR was performed using the cDNA of yeast precursor ALDH2 as a template. The 5′ primer was selected such that it can exclude the leader portion. The resulting PCR products were digested with *Nde I* and *BamH I* and were insert into pRS316M/yALDH2 in which yALDH2 fragment has been removed by digesting with *Nde I/BamH I*. The plasmid was confirmed by sequencing and termed as pRS316M/yALDH2.

2.5. Expression and purification of Yeast cytosolic and mitochondrial ALDH

The wild type and the mutant yeast ALDHs in pET24a plasmid were expressed in Bl21 cells. The cells were harvested and lysed in buffer A (50 mM phosphate buffer, pH 7.5, 500 mM NaCl, 1 mM BME). After centrifugation protamine sulfate was added to the supernatant to precipitate the DNA. It was centrifuged and the supernatant was applied to a nickel column equilibrated with buffer A. After washing the column with buffer A plus 50mM imidazole, the bound proteins were eluted with a gradient of imidazole (50–500mM) in buffer A. The fractions were assayed for ALDH activity as described previously [5, 16]. The fractions with high ALDH activity were run on SDS-PAGE and the fractions homogenous on SDS-PAGE were pooled and dialysed against buffer B (50 mM phosphate buffer, pH 7.5, 100 mM NaCl, 1 mM BME).

2.6. Cellular Fractionation

Yeast pre-cultures were grown under selective pressure in minimal medium with glucose as carbon source. Aliquots of the pre-cultures were used to inoculate 500 ml cultures of SM medium containing 3% ethanol as carbon source. Cultures were harvested as stationary phase by centrifugation and washed once with distilled water. Cellular fractionation to

produce mitochondrial and cytosolic fractions was conducted as described elsewhere [17,18]. The mitochondria pellets were carefully resuspended in the buffer containing 5 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM PMSF and were lysed by sonication. The supernatant containing the matrix protein was saved and used for assay.

2.7. Protein Analysis and activity measurement

Yeast cells grown in the minimal medium with glucose or ethanol as carbon source were collected. Whole cell protein extracts were prepared using glass bead lysis as described previously [5,19]. Aldehyde dehydrogenase activity was measured as previously described [5]. Units are expressed as nanomoles of NADH or NADPH formed by aldehyde dehydrogenase per milligram protein per minute. Protein concentration was determined by the Bradford dye-binding method (BioRad).

2.8. Immunoblots analysis

Immuonoblot analysis was performed as previously described [5]. Sample of whole cell or subcellular protein extract was electrophoresed on 10% polyacrylamide-SDS gels and transferred to nitro cellulose membrane. The membrane was incubated using 1:1000 dilution of anti yeast cytosolic ALDH1, anti yeast mitochondrial ALDH2 or anti-human cytosolic ALDH1, anti human mitochondrial ALDH2 rabbit antiserum. The immunobands were visualized by colorimetrically as performed previously [5].

2.9. Pulse-Chase and immuno Precipitation

Pulse-chase experiments were performed as described by Toth and Coffino [21] with some modification. Cultures in mid-logarithmic phase (A_{600}= 0.3–0.5) in SM medium with glucose as a carbon source at 30°C were washed once in labeling medium (identical to SM, but without methionine and equimolar NH_4Cl replacing $(NH_4)_2$ SO_4), resuspended in the same medium to a final cell density of 2 at A_{600}, cultured for 30–40 minute and then labeled for 10 minutes by addition of [^{35}S]methionine. Chase was initiated by centrifuging the cells and resuspended them in pre-warmed SM containing unlabeled 1 mM cysteine and 1 mM methionine. Aliquots were collected periodically and immediately washed once with 20 mM NaN_3. Cell extract was prepared by glass bead agitation in 200 μl buffer C (1% Triton X-100, 0.2% SDS, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4). The insoluble material was removed by centrifugation. The supernatant was mixed with 300 μl buffer D (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4) and incubated with 10 μl correspondent polyclonal anti-ALDH antiserum at 4°C overnight. Immune complexes were bound to protein A agarose (Santa Cruz Biotech) by shaking at 4 °C 2 hours. Bound immune complexes were washed twice with Buffer C, once with buffer D and suspended in 30 μl SDS sample buffer and boiled for 5 min to release the proteins. The whole supernatant was run on SDS-PAGE and the labled ALDHs were visualized using a Typhoon phosphorImager system and ImageQuant NT (Molecular Dynamics) software [19,20].

2.10. In Vitro Mitochondrial Import Assay

Mitochondria were isolated from the protease-deficient *S. cerevisiae* strain (ATCC24657) [17,22] and used for import assays as described previously [19,20]. All native precursors and chimeric precursors, such as native yeast ypALDH2, chimeric ypALDH1, human hpALDH2, and chimeric hpALDH1, were inserted into the downstream of T7 promoter at pT7-7 plamids. Radiolabeled proteins were synthesized using the TNT quick Coupled transcription and translation system (Promega). The isolated yeast mitochondria were incubated with the labeled translated pALDHs at 30°C for 20 min. Visualization and analysis of import from SDS-PAGE was performed using a Typhon phosphorImager system (Molecular Dynamics) and ImageQuanNT (Molecular Dynamics) software for data analysis.

3. Results

3.1. The modified cytosolic ALDH1 (yALDH1) with low activity barely support the growth of the triple deleted strain on ethanol

In our previous study it was shown that a double disrupted strain (ΔyALDH2+ ΔyALDH5) of *S. cerevisiae* was able to grow on ethanol while the triple deleted strain (ΔyALDH1+ΔyALDH2+ ΔyALDH5) was unable to grow on ethanol suggesting that the cytosolic yALDH1 might have an important role on ethanol metabolism [5]. To study how the activity of yALDH1 was related to the growth of the deleted strain we expressed the wild type or the mutant forms of yALDH1 in the triple deleted strain to test its ability to grow on ethanol. The yALDH1 is essentially NADP dependent and is activated by Mg^{2+} ions [6,9]. We mutated the critical amino acids in yALDH1 that could interact with NADP to disrupt the NADP binding as well as the activity. The structure of yALDH1 is not known but it shares 50% sequence identity with both the human mitochondrial and cytosolic forms of ALDH, making it possible to simulate the structure of yALDH1 by using the coordinates of human mitochondrial NAD dependent ALDH (hALDH2) [23,24]. UCSF Chimera program was used to build the structures and it appeared that both structures are essentially identical (Figure 1A). From the structure of hALDH2 (Fig. 1B), it appears that the residues K192, E195 and G225 are within 3.5Å from 2′- and 3′-hydroxyls of the adenine ribose in NAD (Fig 1B). These residues correspond to K197, A200 and G228, respectively, in yALDH1. In an attempt to disrupt the NADP binding site of yALDH1, the A200E mutation was made since a negative charged amino acid could repel the phosphate group of NADP.

The A200E mutant of yALDH1 was expressed in *E. coli* and purified to essentially homogeneity using a nickel-affinity column. As a control, native cytosolic yALDH1, also cloned to possess a his-tag, was expressed in *E. coli* and purified in an identical manner. The enzyme activities were measured using propionaldehyde as substrate as reported earlier [5]. Compared to the wild type enzyme (3000U), the mutant had only 2% activity (60U), when NADP was used as coenzyme (Table 1) suggesting that the mutation affected the coenzyme binding site. To further lower the NADP dependent activity of yALDH1, the A200E, V201Q double mutant was made. It appears from the Figure 1B that the residue glutamine in hALDH2 is closer the to ribose ring of NAD compared to valine residue in yALDH1, limiting the space for phosphate group of NADP. The double mutant yALDH1 was expressed in *E. coli* and purified by nickel-affinity chromatography. The activity of the purified double mutant enzyme with NADP as a coenzyme was even lower (12U) than that of the single mutant (60U) but the NAD dependent activity was found to be slightly higher (11U) than the wild type (3U) (Table 1). Thus, we were able to make mutant forms of yALDH1 that had significantly lower activity compared to the wild type and these mutants were expressed in the triple deleted strain separately to study the effect of yALDH1 activities on the growth of the deleted yeast strain on ethanol medium.

The growth of the triple deleted strain transformed with native yALDH1 was almost as good as wild type yeast. However, the growth of the triple deleted strain on ethanol was found to be very poor when it was transformed with the double mutation (A200E, V201Q). In contrast, yeast strain with the single point mutant (A200E) grew much better than the double mutant. The optical density of growth on ethanol as a sole carbon source for wild type, single and double mutant were 2.5, 0.54 and 0.03, respectively. The Western blot analysis of whole cell extracts showed that the wild type and the mutant forms of enzyme were expressed similarly (data not shown). This study showed that the growth of the deleted strain on ethanol depends upon the active NADP dependent yALDH1.

3.2. The growth of the triple deleted yeast strain on ethanol when expressing yeast mitochondrial ALDH (yALDH2) in the cytosol

Since we found that the growth of the deleted strain depends on the NADP dependent yALDH1 in cytosol it was interesting to test if a mitochondrial NAD dependent ALDH2 could support the growth of the deleted strain when it was placed in cytosol. To accomplish this, just the mature portion of cDNA of yALDH2 (without leader peptide), was cloned in a plasmid as described in “Materials and Methods” section. The triple deleted strain was transformed with the plasmid and cells were first grown in SC medium with glucose to test if the mature ALDH2 was expressed. Western blotting of whole cell extracts using the antibody against mitochondrial ALDH2 showed that the mature form was expressed as shown in lane 2, Figure 2A. As a positive control the deleted strain was also transformed with the plasmid containing the precursor cDNA of ypALDH2 (with leader peptide). Western blotting of whole cell extracts using anti-mitochondrial ALDH2 showed that protein was expressed to a similar extent as was the mature (Figure 2A lane 3). Therefore, the two forms of mitochondrial ALDH2 were expressed similarly in the deleted strain. The deleted strain expressing mitochondrial mature or precursor ALDH2 was found to grow on ethanol as sole source of carbon either on plates (Figure 3A) or liquid media (data not shown). Western blot analysis after cell fractionation from cells expressing yALDH2 showed that mature ALDH2 was expressed and it remained in the cytosol (Figure 2B.1). The deleted strain expressing the precursor form of mitochondrial ALDH2 was also subjected to cell fractionation studies as we did previously [5,19]. The protein was found in the matrix space as expected (Figure 2B.2). It was found that the whole cell extract of deleted strain either expressing mature or precursor form of ALDH2 had similar activities when propionaldehyde was used as substrate (Table 2). The data leads us to suggest that mitochondrial ALDH2 which is primarily a NAD requiring enzyme was active in cytosol and could support the growth of deleted strain on ethanol medium.

3.3. The growth of triple deleted yeast strain expressing human mitochondrial ALDH(hALDH2) in cytosol

Since yeast mitochondrial ALDH2 possesses some NADP dependent activity [5], human mitochondrial ALDH2 (hALDH2) which is essentially a NAD-dependent enzyme, was designed to be placed in cytosol to study if hALDH2 could support the growth of deleted yeast strain on ethanol medium. The deleted yeast strain was transformed with the plasmid containing the cDNA of mature hALDH2 so that it would remain in cytosol. The deleted strain was also transformed with the plasmid containing the cDNA of precursor hpALDH2 The cells were grown in SC medium to test that the proteins were expressed. Western blot analysis of whole cell extracts with human ALDH2 antibody showed that the hALDH2 was expressed in the deleted strain (Figure 4A). The deleted yeast strain was able to grow on ethanol when either precursor or mature form of human ALDH2 was expressed (Figure 3B). ALDH activities from whole cell extracts either expressing mature or precursor human ALDH2 were also found to be similar (Table 2). To show that the either hALDH2 was located properly in the deleted strains, Western blot analysis after sub cellular fractionations with hALDH2 antibody was performed. It was found that the mature ALDH2 was located in cytosol of the deleted strain as expected (Figure 4B.1) while precursor form was located in mitochondria and presumably was processed by mitochondrial processing peptidase (Figure 4B. 2). This showed that a strictly NAD- dependent hALDH2, when placed in cytosol, can replace the yeast cytosolic NADP dependent ALDH1 to support the growth of triple deleted yeast strain on ethanol.

3.4. Growth of triple deleted strain on ethanol when cytosolic form of ALDH (ALDH1) was expressed in mitochondria

To determine if the NADP dependent yALDH1 would function in mitochondria and could support the growth of triple deleted strain on ethanol, a cDNA corresponding to the leader peptide of mitochondrial pALDH2 was fused to the cDNA of cytosolic yALDH1 and it was inserted into the plasmid. Similarly, cDNA of the leader peptide from precursor human pALDH2 was fused to cDNA of cytosolic hALDH1 and was inserted into plasmid to test if a hALDH1 was active in yeast mitochondria. The constructs are shown schematically in Figure 5A. Triple deleted yeast strain was transformed with both plasmids separately and it was found that neither construct could support the growth of the deleted strain on ethanol as sole source of carbon (Figure 3A, and B). Finding no growth could be the result of several factors such as the protein was not synthesized, the fusion protein could be degraded or cytosolic ALDH1 was not active in mitochondria. To test that the fusion protein was synthesized, after transformation, cells were grown in SC medium with glucose as a carbon source. Whole cell extract was used for Western blot analysis with anti cytosolic yeast or human cytosolic ALDH1 antibody. No bands were detected after Western blotting (Lanes 1 in Figure 5B and C) suggesting that either the protein was not synthesized or the protein was degraded rapidly. However, the deleted strain could grow on ethanol when transformed with the cDNA of either native cytosolic ALDH1 from yeast or human (Figure 3A, and B). Western blot analysis of whole cell extracts either expressing yeast or human cytosolic ALDHs with yeast or human antibody showed that the cytosolic protein was expressed (Lanes 2 in Figure 5B and C). To examine the fate of the fusion protein a pulse chase experiment was performed as described in “Materials and Methods” section. Pulse chase studies showed that fusion proteins (leader peptide fused to cytosolic ALDH1) either from yeast or human was degraded within 10 minutes after synthesis (Figure 6). The mitochondrial precursor forms from yeast and human, however, were stable for at least 90 minutes as shown in Figure 6.

The fusion protein could be degraded either in cytosol or in mitochondria. To test this, an *in vitro* import assay was performed to determine the import competency of the fusion proteins. The radio labeled fusion proteins were incubated with isolated yeast mitochondria as described in “Materials and Methods”. The native mitochondrial precursor from of yeast and human pALDH2 were also included in the import study. The native precursor forms of mitochondrial pALDH2 from both yeast and human were efficiently translocated to mitochondria (Figure 7A and B). However, insignificant amount of either fusion protein was found to be translocated to mitochondria (Figure 7A and B) suggesting most of the fusion protein was degraded in cytosol. Because of the rapid degradation of the fusion protein, it was not possible to study the role of the cytosolic form in mitochondria as we did for mitochondrial form of ALDH.

4. Discussion

During the growth of *S. cerevisiae* on glucose, pyruvate is produced through glycolysis which can be directed to either ethanol fermentation or respiration based on the amount of glucose present in the medium. In *S. cerevisiae* fermentation can occur even in the presence of oxygen. After depletion of glucose and accumulation of ethanol, yeast use the product ethanol as substrate in aerobic condition. Acetate is produced by the action of ADH and ALDHs from ethanol. In our previous study we found that the double disrupted strain ΔALDH1+ΔALDH2 or the triple disrupted strain grew slowly in glucose and were unable to grow on ethanol medium [5] but the growth was restored by the addition of acetate which showed that ALDH1 and ALDH2 are important for growth.

Interestingly, unlike human cytosolic and mitochondrial ALDH, yeast cytosolic and mitochondrial ALDHs have very similar Km values for acetaldehyde. However, it may be possible that the yALDH1 could oxidize bulk of the acetaldehyde produced in cytoplasm since YALDH1 uses NADP while the previous step with ADH is NAD dependent, avoiding a demand in the pool of just one kind of coenzyme in cytosol. In addition, steps involved in ADH and yALDH1 both occur in cytosol. It was, therefore, interesting to test if a NAD dependent mitochondrial yALDH2 could replace a NADP dependent cytosolic yALDH1 to support the growth of the deleted strain in ethanol.

First, the mutant forms of yALDH1 were made to determine the effect of NADP dependent ALDH activity on the growth of the deleted strain in ethanol medium. The NADP binding site of yALDH1 was disrupted by creating the single (A200E) and double mutant (A200E, V201Q) that reduce the NADP dependent activity of cytosolic ALDH1 by 50 and 300 fold, respectively. The low ALDH activity of the double mutant was found to be not sufficient to support the growth of triple deleted strain on ethanol as sole carbon source. However, the triple deleted strain was able to grow on ethanol when transformed with native yALDH1 suggesting that the growth on ethanol depends upon the activity of NADP dependent yALDH1.

Next, we employed another strategy that allowed us to investigate the importance of coenzyme specificity of yALDH1 on the growth of the deleted strain in ethanol medium. It was possible for us to transform the triple deleted strain with either yeast or human mature mitochondrial (without leader peptide) ALDH2 which are primarily NAD dependent enzymes. Both yeast and human mitochondrial ALDH2 were found to be stable in cytosol and were able to support the growth of deleted strain in ethanol. This finding show that the coenzyme specificity was not the dominating driving force to have the native enzyme be a NADP-dependent. Further, the finding that the triple deleted strain expressing mitochondrial ALDH2 in cytosol could grow well in ethanol shows that the subcellular localization of the enzyme is not an important factor, at least in yeast.

It was of interest to find that while the yeast or human mitochondrial ALDH2 was stable in cytosol, the opposite was not true. The cytosolic isozyme when engineered to have a leader sequence at its N-termini was found to be rapidly degraded. *In vitro* import study showed that the fusion protein was poorly imported into mitochondria suggesting it could be degraded in the cytosol. Apparently the presence of the leader slows down folding and allows proteolysis to take place in cytosol. In the case of native precursor protein, the import rate is very fast resulting in minimum proteolysis in cytosol [25]. To import a preprotien into mitochondria, it needs to form a structure compatible enough to interact with the mitochondrial protein import receptors [25,26]. It is possible that the addition of leader peptide to the N-terminus of cytosolic ALDH1 folds it into a conformation not suitable for import and thus degraded readily. To be active an enzyme needs to fold properly. It appears that the mature mitochondrial isozyme can fold in cytosol but that proteolysis of the cytosolic form containing leader peptide is faster than import into mitochondria. It is not known if the folding of ALDH requires the action of a heat shock protein, but if it does, then the recognition features for folding of at least the mitochondrial isozymes are present in the mature portion of the molecule. Earlier work in our laboratory suggested that the information for folding might reside in the N-terminal part of the mature portion of the enzyme [27].

This study does not answer the question as to why some isozymes of ALDH evolved to use NAD while others use NADP as the coenzyme. Here it is found that mitochondrial NAD dependent ALDH2 can substitute cytoplasmic NADP dependent ALDH1 suggesting that the

coenzyme pool or specificity in cytosol might not be a deciding factor in acetaldehyde oxidation but it could be important for metabolism of other substrates

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Abbreviation

yALDH1	yeast cytosolic aldehyde dehydrogenase
yALDH2	yeast mitochondrial aldehyde dehydrogenase
ypALDH2	yeast mitochondrial precursor aldehyde dehydrogenase
ypALDH1	yeast cytosolic ALDH1 with mitochondrial leader peptide fused to the N-terminus
hALDH1	human cytosolic aldehyde dehydrogenase
hALDH2	human mitochondrial aldehyde dehydrogenase
hpALDH2	human mitochondrial precursor aldehyde dehydrogenase
hpALDH1	human cytosolic ALDH1 with mitochondrial leader peptide fused to the N-terminus

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General Significance

The present study provides a basis to understand the coenzyme preference of ALDH in ethanol metabolism in yeast.

Highlights

- Mutant ALDH1 barely support the growth of triple ALDH deleted yeast on ethanol
- NAD dependent Mitochondrial ALDH2 replaces cytoplasmic NADP dependent ALDH1 in yeast
- ALDH1 with mitochondrial leader attached is unable to be imported into mitochondria
- Localization or coenzyme specificity of ALDH is not essential for aerobic growth of yeast on ethanol

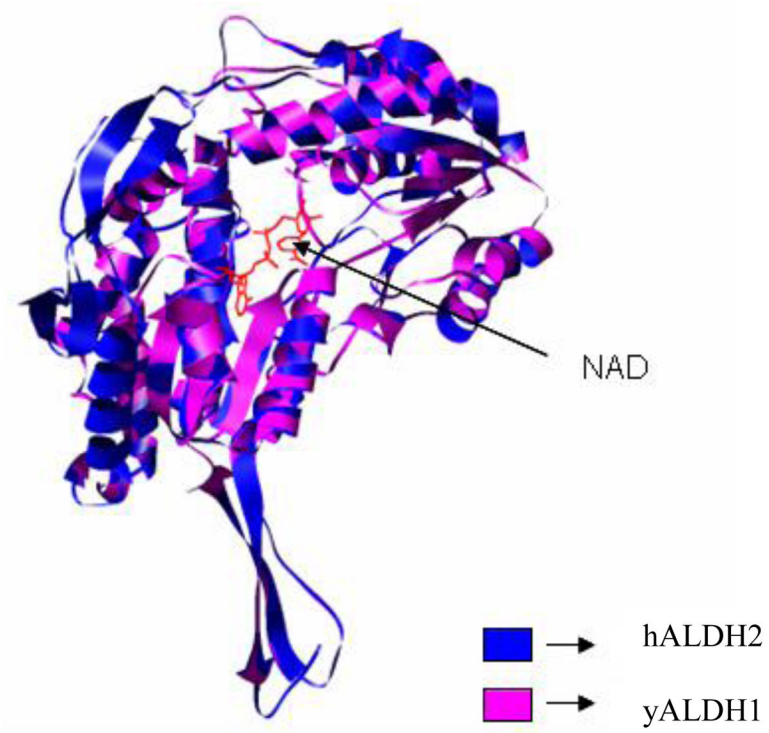

Figure 1B.

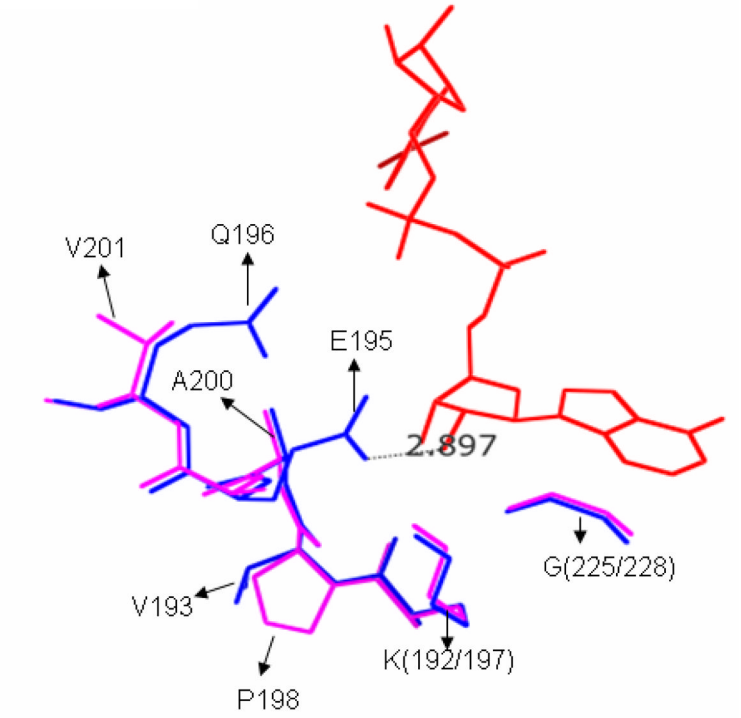

Figure 1.

A. The structure of human ALDH2 (blue) with NAD (red) (PDB 1CW3). The structure of yeast ALDH1 was built using the coordinates of human ALDH2 using the program UCSF chimera (http://www.cgl.ucsf.edu/chimera/).
B. Location of amino acids of human ALDH2 (blue) and yeast cytosolic ALDH1 (magenta) which are close to the 2 and 3 hydroxyl group of ribose ring of NAD (red). The glutamic acid (E195) from human ALDH2 is within 3Å from the hydroxyl group of ribose of NAD (red).

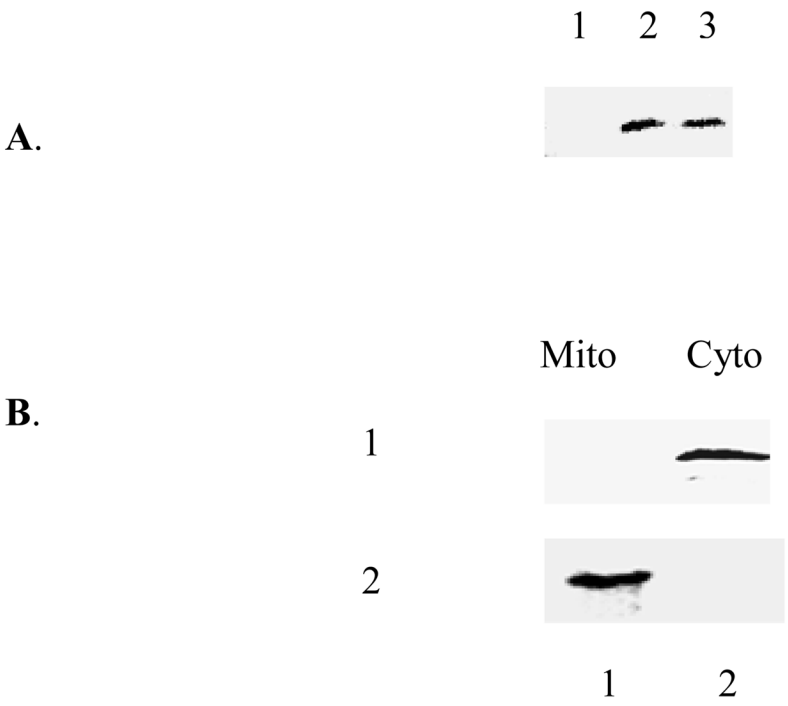

Figure 2.

The deleted yeast strain expressing yeast precursor and mature forms of mitochondrial ALDH2.

A. The immunoblots of whole cell extracts of triple deleted yeast strain expressing yeast ALDHs when grown in glucose medium. Lane 1 is the deleted strain with empty vector, lane 2 is deleted strain expressing mature mitochondrial ALDH2, lane 3 is deleted strain expressing the mitochondrial precursor ALDH2. Antibody against yeast mitochondrial ALDH2 was used. **B**. The immunoblots of cytosolic and mitochondrial fractions of triple deleted yeast strain expressing yeast ALDHs grown on ethanol as sole carbon source. 1. The deleted strain expressing the mitochondrial mature form of ALDH2. 2. The mitochondrial precursor form of ALDH2 was expressed in the deleted strain. Lane 1 and 2 are the mitochondrial and cytosolic fractions, respectively.

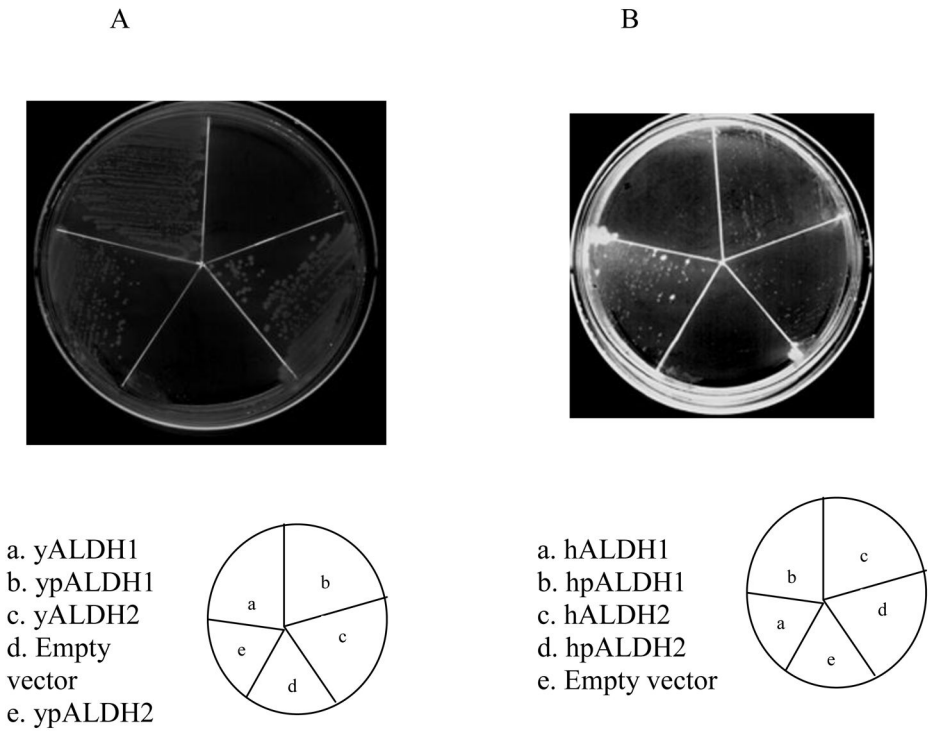

Figure 3.
Growths of the deleted strain expressing yeast (A) and human (B) ALDHs on ethanol medium. pALDH2- mitochondrial precursor ALDH2, ALDH2- mitochondrial mature ALDH, ALDH1-cytosolic ALDH, pALDH1 -mitochondrial leader fused to cytosolic ALDH1.

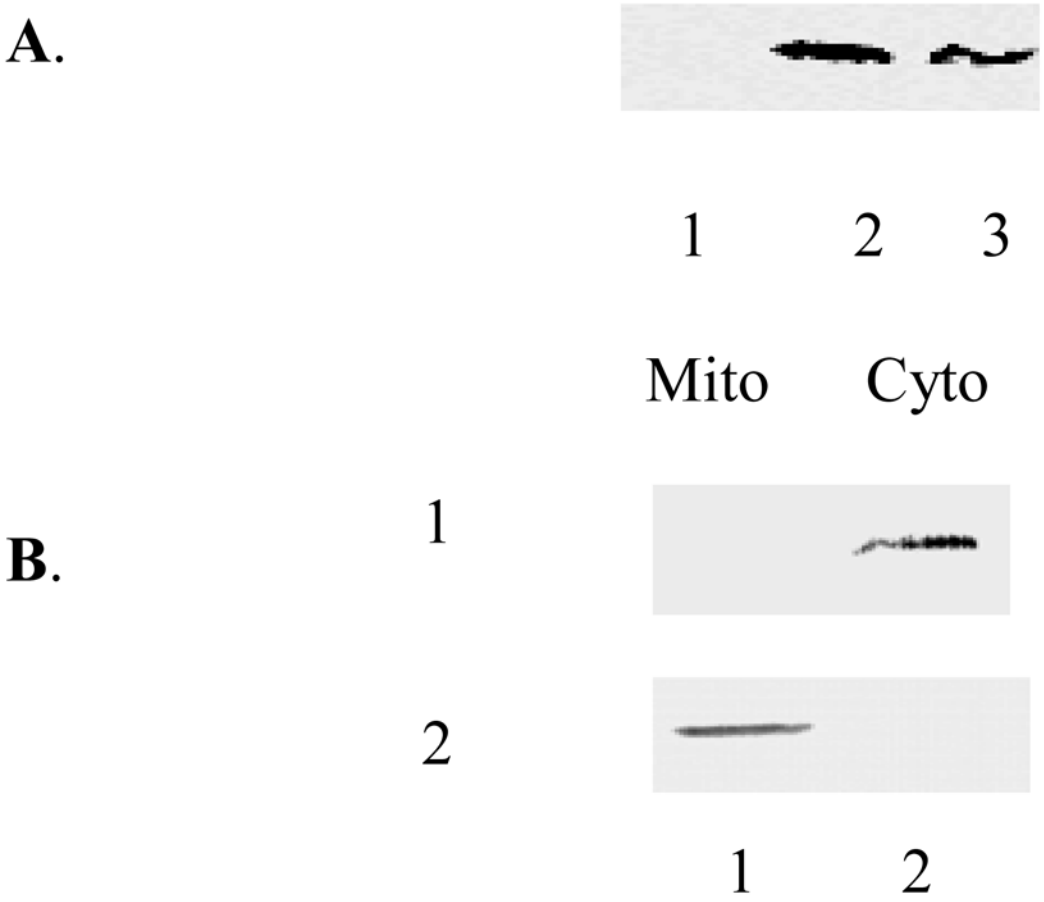

Figure 4.
The deleted yeast strain expressing human precursor and mature forms of mitochondrial ALDHs.
A. The immunoblot of whole cell extracts of deleted strain expressing human ALDHs when grown in glucose medium. Lane 1 is the deleted strain with empty vector, lane 2 is deleted strain expressing mature mitochondrial ALDH2, lane 3 is deleted strain expressing the mitochondrial precursor pALDH2. Antibody against human mitochondrial ALDH2 was used. **B**. The immunoblots of cytosolic and mitochondrial fractions of triple deleted yeast strain expressing human ALDHs grown on ethanol as sole carbon source. 1. The deleted strain expressing the mitochondrial mature form of ALDH2. 2. The mitochondrial precursor form of ALDH2 was expressed in the deleted strain. Lane 1 and 2 are the mitochondrial (mito) and cytosolic (cyto) fractions, respectively.

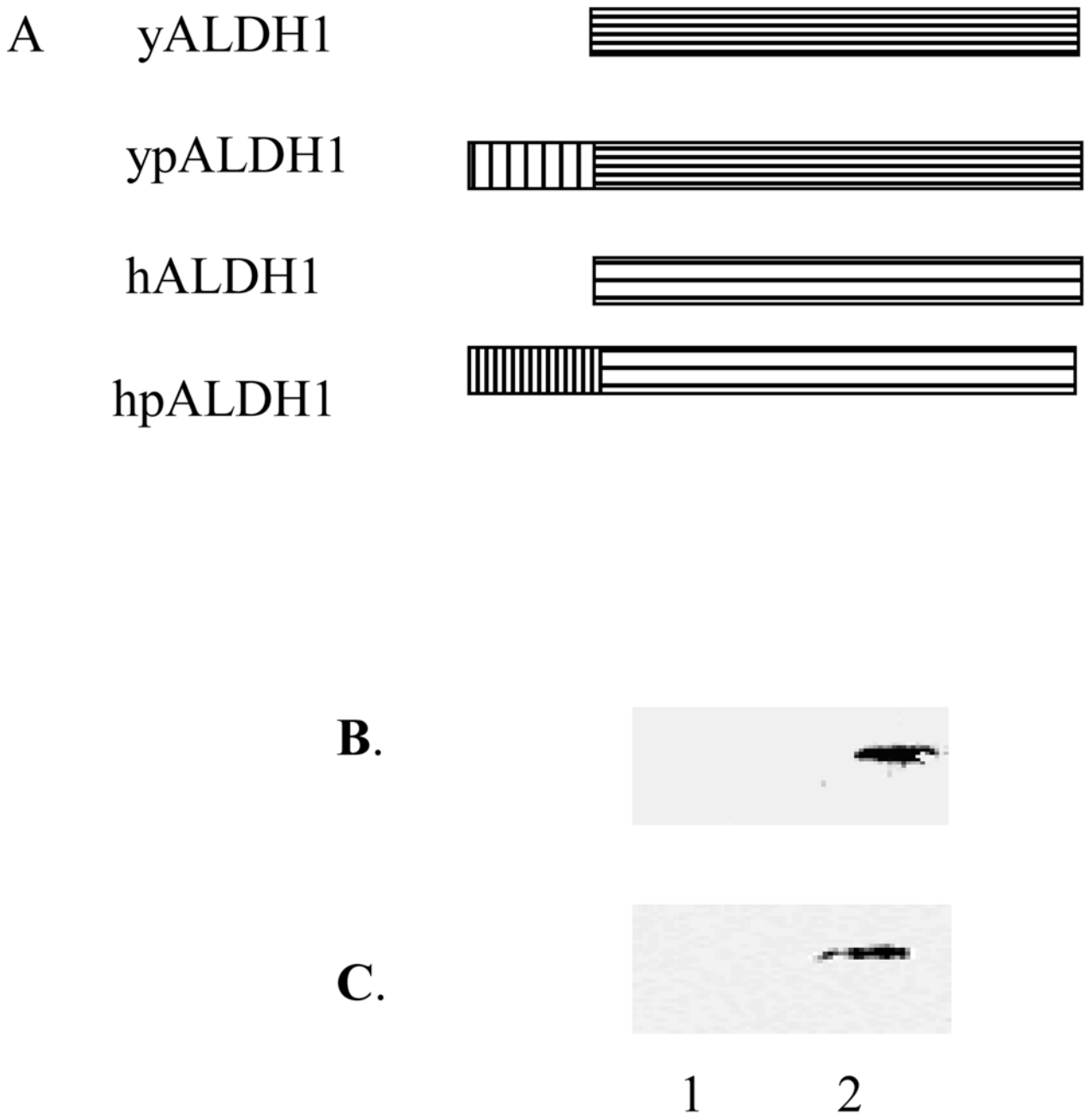

Figure 5.

The deleted yeast strain expressing the chimeric proteins, mitochondrial leader sequence of pALDH2is fused to the cytosolic ALDH1.

A. The constructs are shown schematically. The yeast mitochondrial leader peptide of pALDH2 ( ) is fused to the N-terminus of yeast cytosolic ( ) ALDH1, and the human mitochondrial leader peptide of pALDH2 ( ) is fused to the human cytosolic ( ) ALDH1.

B. The immunoblots of whole cell extracts of deleted strain expressing yeast cytosolic or chimeric ALDHs when grown in glucose medium. Lane 1 is for chimeric ALDH and lane 2 is for cytosolic ALDH. Antibody against yeast cytosolic ALDH was used.

C. The immunoblots of whole cell extracts of deleted strain expressing human cytosolic or chimeric ALDHs when grown in glucose medium. Lane 1 is for chimeric ALDH and lane 2 is for cytosolic ALDH. Antibody against human cytosolic ALDH was used.

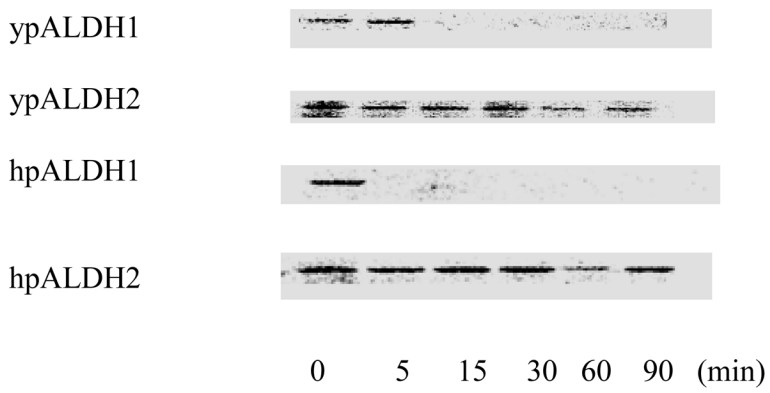

Figure 6.
Pulse chase experiments to determine the turnover of radio labeled yeast native precursor and chimeric and human native precursor and chimeric ALDH in the deleted yeast strain. The data showed that in both cases the chimeric ALDHs were degraded within few minutes after synthesis while the native ALDHs were found to be stable.

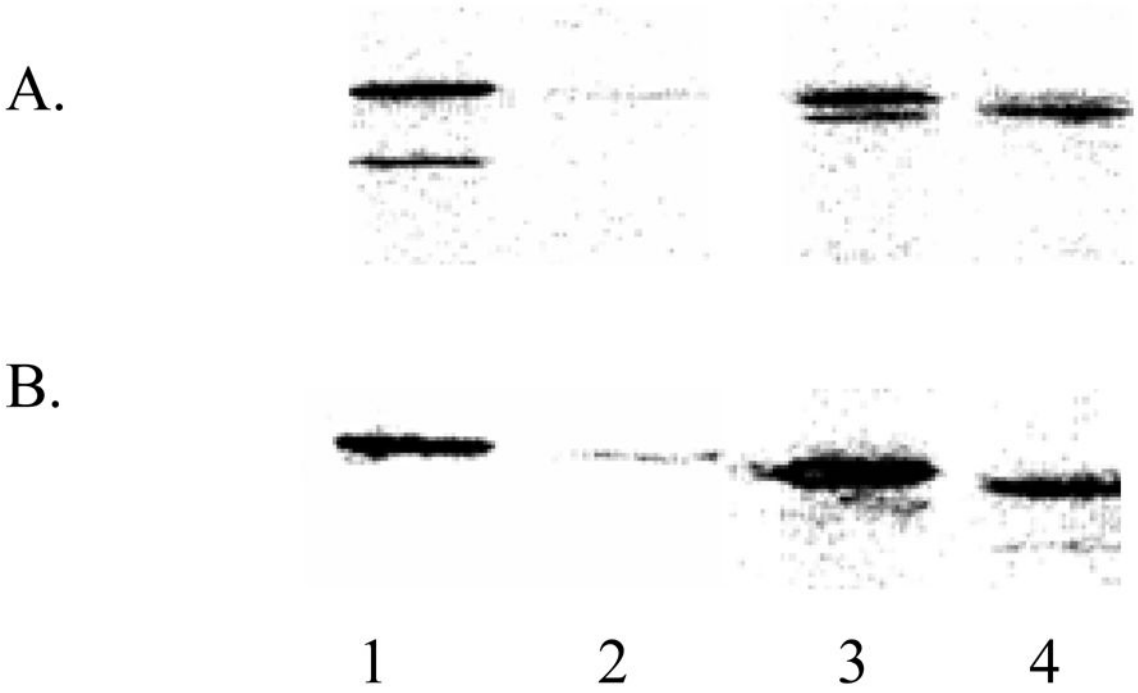

Figure 7.

In vitro import of radio labeled ALDHs from yeast (A) and human (B) in yeast mitochondria. Radio labeled proteins were incubated with yeast mitochondria for 30 minutes. Proteinase K was added to digest the non importable ALDHs and mitochondria was recovered by using centrifugation. Mitochondrial proteins were separated on SDS-PAGE and proteins were visualized using phosphor imager as described in “Materials and Methods”. Lane 1 is for the translated chimeric protein (mitochondrial leader fused to cytosolic ALDH), lane 2 is for import of the chimeric protein, lane 3 is for translated mitochondrial precursor protein and lane 4 is import of the precursor protein.

Table 1

Specific activities of native, single and double mutant yeast cytosolic ALDH (yALDH1).

yALDH1	Specific activity		Activity ratio (NADP/NAD)
	NADP	NAD	
Native	3000	3.12	892
A200E	60	3.15	17.4
A200E, V201Q	12	11.3	1.03

Specific activity assay were performed at *V*max condition with propionaldehyde as substrate. Units are expressed as nano moles of NADH or NADPH formed by aldehyde dehydrogenase per milligram protein per minute. Values represent averages of three independent determinations.

Table 2

Aldehyde dehydrogenase activity in whole cell protein extracts following transformation of the triple ALDHs disrupted yeast strain with single copy plasmids.

Enzymes	Specific activity[a]
yALDH1	50
ypALDH1	ND[b]
yALDH2	70
ypALDH2	60
hALDH1	6.0
hpALDH1	ND[b]
hALDH2	3.0
hpALDH2	3.5

[a] Specific activity assay were performed at *V*max condition with propionaldehyde as substrate. NAD was added for obtaining human ALDH activity and NADP for yeast ALDH. Values represent averages of three independent determinations. Protein extracts were prepared from cultures grown in 3% ethanol medium.

[b] none detected.