Human liver mitochondrial aldehyde dehydrogenase:
Three-dimensional structure and the restoration
of solubility and activity of chimeric forms

LI NI, 1,3 JIANZHONG ZHOU, 1,3 THOMAS D. HURLEY, 2 AND HENRY WEINER 1
1 Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153
2 Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202-5122
(Rceived June 15, 1999; Accepted October 6, 1999)

Abstract
Human liver cytosolic and mitochondrial isozymes of aldehyde dehydrogenase share 70% sequence identity. However, the first 21 residues are not conserved between the human isozymes (15% identity). The three-dimensional structures of the beef mitochondrial and sheep cytosolic forms have virtually identical three-dimensional structures. Here, we solved the structure of the human mitochondrial enzyme and found it to be identical to the beef enzyme. The first 21 residues are found on the surface of the enzyme and make no contact with other subunits in the tetramer. A pair of chimeric enzymes between the human isozymes was made. Each chimera had the first 21 residues from one isozyme and the remaining 479 from the other. When the first 21 residues were from the mitochondrial isozyme, an enzyme with cytosolic-like properties was produced. The other was expressed but was insoluble. It was possible to restore solubility and activity to the chimera that had the first 21 cytosolic residues fused to the mitochondrial ones by making point mutations to residues at the N-terminal end. When residue 19 was changed from tyrosine to a cysteine, the residue found and activity to the chimera that had the first 21 cytosolic residues fused to the mitochondrial ones by making point mutations to residues at the N-terminal end. When residue 19 was changed from tyrosine to a cysteine, the residue found in the mitochondrial form, an active enzyme could be made though the $K_m$ for NAD$^+$ was 35 times higher than the native mitochondrial isozyme and the specific activity was reduced by 75%. This residue interacts with residue 203, a nonconserved, nonactive site residue. A mutation of residue 18, which also interacts with 203, restored solubility, but not activity. Mutation to residue 15, which interacts with 104, also restored solubility but not activity. It appears that to have a soluble or active enzyme a favorable interaction must occur between a residue in a surface loop and a residue elsewhere in the molecule even though neither make contact with the active site region of the enzyme.

Keywords: aldehyde dehydrogenase; chimeric proteins; isozymes; restoration of activity; solubility; three-dimensional structure

Many forms of human aldehyde dehydrogenase (ALDH) have been identified and some characterized. The mitochondrial and cytosolic forms, called class 2 (ALDH2) and class 1 (ALDH1), respectively, have been perhaps the best studied. The primary sequences reveal that they share nearly 70% identity (Hempel et al., 1985; Hsu et al., 1985). The three-dimensional structures of the beef ALDH2 (Steinmetz et al., 1997) and sheep ALDH1 (Moore et al., 1998) enzymes were recently determined and the structures were found to be very similar. Since the sheep and human ALDH1 enzymes share 95% sequence identity, as does the ALDH2 pair, it can be assumed that structure of the human cytosolic isozyme will be virtually identical to the structure of the corresponding sheep form. In this paper, we will show that the structure of the human ALDH2 enzyme is virtually identical to that of the beef enzyme.

The 70% sequence identity between the class 1 and 2 isozymes of ALDH is found through out the proteins except within the first 21 amino acids that are coded by the first exon (Guan & Weiner, 1990). These 21 residues exclude the leader sequence portion of the mitochondrial isozyme that also is coded by the first exon. Only three amino acids, Ser1, Thr5, and Pro9, are conserved between the two isozymes in this region of the proteins. The three-dimensional structures show that amino acids 7–21 are on the surface of the enzyme and are not in contact with other subunits; the first six residues are apparently disordered and were not located in either X-ray structure (Steinmetz et al., 1997; Moore et al., 1998).

Chimeric proteins between ALDH1 and ALDH2 were prepared by fusing the first 21 amino acids from one isozyme to the remaining 479 of the other. Unexpectedly, only one of the recombinantly expressed forms was soluble. Here, we report the three-dimensional structure of the recombinant human ALDH2 isozyme complexed with cofactor and Mn$^{2+}$ ions. Using the newly ob-
tained structural information, we attempted to design mutational substitutions to restored solubility and activity to the chimeric form that possessed the first 21 residues of the cytosolic enzyme.

**Results**

The structure of the human ALDH2 isozyme was solved by molecular replacement using the beef isozyme (Steinmetz et al., 1997) as the search model and is essentially identical to it. When aligned, the two ALDH2 isozymes exhibit an overall root-mean-square difference (RMSD) of 0.3 Å for all α-carbon positions in a tetramer. The crystallographic parameters are tabulated in Table 1. The final refined human ALDH2 model contains eight identical chains per asymmetric unit arranged as two independent tetramers. Each subunit contains a bound NAD$^+$ molecule and Mn$^{2+}$ ion, although only three subunits exhibit electron density for the nicotinamide ring similar to what was reported for the beef enzyme (Steinmetz et al., 1997). The other subunits exhibit electron density for the nicotinamide ring that is similar to that reported for the sheep cytosolic enzyme (Moore et al., 1998). The Mn$^{2+}$ ion is bound directly to the NAD$^+$ molecule through the adenosine phosphate, as was observed for the Sm$^{3+}$ ion in the beef coenzyme complex (Steinmetz et al., 1997).

A pair of chimeric enzymes was prepared where the first 21 residues from one isozyme were attached to the remaining 479 of the other. Chimera 1, which possessed only the first 21 residues of the mitochondrial isozyme fused to the remaining 479 amino acids from the cytosolic isozyme, was active, while the counterpart, chimera 2, was insoluble. The active chimera was purified to homogeneity and partially characterized with respect to some basic kinetic properties, as shown in Table 2.

The kinetic properties of chimera 1 were found to be very similar to those of the class 1 enzyme. This was not totally unexpected, since the construct was primarily a class 1-like enzyme. These first 21 amino acids in the native form of the enzyme lie on the surface of the subunit, making no contact with other subunits in the tetramer (Fig. 1). The exact positions of the first seven residues have not been established for either isozyme, and they are assumed to have freedom of motion. The conformation of the peptide possessing amino acids 7–21 is shown in Figure 2. Assuming that the

### Table 1. Data collection statistics and refinement statistics for human mitochondrial aldehyde dehydrogenase

<table>
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<tr>
<td>Space group</td>
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<tr>
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<td>Resolution range</td>
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<td>Total observations</td>
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<td>Completeness (%)</td>
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<td>$I/\sigma_I$ (12.6 (3.0°))</td>
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<tr>
<td>$R_{merge}$ (%)</td>
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<th>Refinement statistics</th>
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<td>Molecules per asymmetric unit</td>
<td>2 tetramers</td>
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<tr>
<td>Resolution range</td>
<td>45.2–5.8 Å</td>
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<td>$R_{free}$ (%)</td>
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<td>RMSD for main-chain atoms (Å)</td>
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<td>0.007</td>
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<td>RMSD from ideal bond angles (°)</td>
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*Values in parentheses indicate the statistics for the highest resolution shell (2.68–2.58 Å).

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**Table 2. Kinetic data for various constructs of aldehyde dehydrogenase**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ NAD$^+$ (µM)</th>
<th>$K_m$ propionaldehyde (µM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
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<tr>
<td>ALDH 1</td>
<td>4.0</td>
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<td>74</td>
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<td>ALDH 2</td>
<td>26</td>
<td>0.5</td>
<td>190</td>
</tr>
<tr>
<td>ALDH 2 Y203H$^b$</td>
<td>30</td>
<td>ND$^c$</td>
<td>140</td>
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<tr>
<td>Chimera 1$^d$</td>
<td>1.1</td>
<td>2.2</td>
<td>71</td>
</tr>
<tr>
<td>Chimera 2 Y203H$^{b/}$</td>
<td>76</td>
<td>0.2</td>
<td>94</td>
</tr>
<tr>
<td>Chimera 2 Y19C$^e$</td>
<td>150</td>
<td>52</td>
<td>45</td>
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</tbody>
</table>

$^a$The values for $K_m$ (NAD$^+$), $K_m$ (propionaldehyde), and $k_{cat}$ were the averages of at least three assays. The relative deviation was ±5%.
$^b$203H is the residue found in the cytosolic isozyme.
$^c$ND, not determined.
$^d$Chimera 1 is the cytosolic enzyme with the first 21 amino acids of the mitochondrial enzyme.
$^e$Chimera 2 is the mitochondrial enzyme with the first 21 amino acids of the cytosolic enzyme.
$^{19}C$ is the residue found in the mitochondrial isozyme.

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**Fig. 1.** The structure of a subunit of human mitochondrial aldehyde dehydrogenase represented as a ribbon diagram. The positions of residues 7–21 are shown along with residue 203 and the active site nucleophile Cys302 and the bound NAD$^+$. All figures were produced using MOLSCRIPT (Kraulis, 1991).
conformation of these two strings of amino acids did not change in
the chimeric construct from what they were in the native structure,
it is not apparent why chimera 2 was not soluble.

An inspection of the class 1 and 2 ALDH structures in the
vicinity of residues 7–21 suggests that there are two regions of
contact between these residues and the rest of the enzyme that
appear to be most critical for the stability of this region of the
enzyme. These are residues 15/104 and 18,19/203. The exchange
of Leu (ALDH1) for Pro (ALDH2) at position 15 and its inter-
action with the residue at position 104 would result in a displace-
ment of this portion of the polypeptide away from the surface of
the enzyme in the ALDH1 structure (Fig. 2). Second, the exchange
of residues at positions 18 and 19 would create a different contact
environment with the residue at position 203 (Fig. 2). In the human
and beef ALDH2 enzymes, residues 17–19 are hydrophobic and
their side chains interact with a hydrophobic patch surrounding
residue 203 (Tyr), but in the ALDH1 structure, both residues 18
(Gln) and 203 (His) are hydrophilic. If these side-chain mediated
contacts were important to the overall structure of the enzyme, it
might be possible to regain solubility, and hence, activity with the
inactive chimera 2 by making changes that would restore these
favorable interactions. Mutating Ala104 in the mitochondrial por-
tion of chimera 2 to a threonine, the residue found in the cytosolic
enzyme, did indeed produce a soluble enzyme. Similarly, changing
the Tyr at position 203 in chimera 2 to a His also produced a
soluble enzyme. Point mutations to residues 18 and 19 of the
cytosolic portion of chimera 2 were also made to create the local
sequence found in the mitochondrial enzyme. The Q18F variant
remained insoluble but the Y19C mutant became soluble (Table 3).
The latter two were purified to homogeneity and some basic kinetic parameters were deter-
imined and are presented in Table 2. As a control, Tyr203 in mi-
тоchondrial enzyme was mutated to a histidine so the enzyme
would possess the residue found in the cytosolic enzyme. It can be
noted that the specific activities of the restored chimera 2 were less
than that of the native mitochondrial isozyme. The Y19C form,
which still has the normal mitochondrial tyrosine at position 203,
proved to be the least active enzyme having just 25% specific
activity and exhibited a large increase in its $K_m$ for NAD$^+$
and for propionaldehyde compared to the native enzyme. The mutations
made to chimera 2 that restored solubility and activity did not
bring the level of activity of the chimera to that of chimera 1. The
latter had properties virtually indistinguishable from the parent
cytosolic form.

Discussion

Reports in the literature vary as to the success investigators had
when using chimeric proteins. Some studies show that soluble
active enzymes can be produced (Kitajima et al., 1990; Carriere
et al., 1997; Mockus et al., 1997; Ahmad et al., 1998; Tasara et al.,
1999) while others state that they could not be made (Friesen,
1996; Gora et al., 1999). When a soluble and active enzyme could
be made, much information about critical interactions can be
obtained.

To better identify the structural environment of residues 7–21 in
the human mitochondrial isozyme, we solved its three-dimensional
structure. Though we originally solved the structure of the beef
enzyme, the latter was never cloned. We find that the structures of
the two mitochondrial forms of ALDH are identical. The major
difference between human and beef mitochondrial ALDH was that
the asymmetric unit possessed a pair of tetramers, and two distinct
conformations were observed for the bound cofactor molecules in the eight subunits. Three of the subunits in the asymmetric unit exhibit a similar conformation for the cofactor as found in the beef ALDH2 structure (Steinmetz et al., 1997), while the other five subunits exhibit a cofactor conformation similar to that reported for the sheep cytosolic isozyme (Moore et al., 1998). Each of the modeled cofactor conformations in the subunits represents the most highly occupied conformation for each subunit. However, all the bound cofactors have high temperature factors and probably exist in both conformations in all subunits, but at different relative occupancies. It appears that disorder in the binding of NAD$^+$ is a common feature of the five mammalian ALDH isozymes whose structures are known (Liu et al., 1997; Steinmetz et al., 1997; Johansson et al., 1998; Moore et al., 1998; Lamb & Newcomer, 1999).

In an attempt to understand the importance of the first 21 amino acids, chimeric proteins were constructed. It is those residues that show the least degree of sequence identity between the two classes of ALDH. Chimera 1, whose first 21 residues were from the mitochondrial enzyme with the remaining 479 from the cytosolic isozyme was active and possessed properties virtually identical to the native cytosolic enzyme. This was not unexpected since these first 21 amino acids are on the surface of the subunit and are not in contact with the active site region (Fig. 1). What was unexpected was that the opposite construct was insoluble. When isolating the enzyme from horse (Johansson et al., 1988) or human (Hempel et al., 1985), a ragged N-terminal sequence was found, suggesting that these first few residues were not important for activity of the enzyme. Presumably, nonspecific proteases removed some amino acids while the enzyme was being purified from tissue extracts. Though the mitochondrial isozyme can function when some of the N-terminal residues were missing, it appears not to tolerate the presence of the 21 residues from the cytosolic isozyme.

There appear to be two critical contact regions between residues 7–21 and the remainder of the enzyme. These contact regions are formed by the interactions between residues 15 and 104 and residues 18, 19, and residue 203 (Fig. 2). From the mutational work performed, it can be concluded that the interaction between residues 19 and 203 was critical to the solubility of the enzyme. When the corresponding residues were from the same isozyme, it was possible to produce enzymes with 25 and 50% of the activity exhibited by the native enzymes. When the Tyr203 in chimera 2 was changed to His, the residue found in the cytosolic enzyme, an active enzyme was produced. Similarly, by changing Tyr19 to Cys, the residue found in the mitochondrial enzyme, an active enzyme also was produced. Thus, the interaction between residues 19 and 203 is needed to maintain the enzyme in an active conformation. The structures of these chimeras and the mutants have not been determined. Neither of these residues comes in direct contact with the active site region of the enzyme. The characteristics of the contacts between residue 203 and residues 18 and 19 differ in the two human ALDH isozymes. In the mitochondrial isozyme residues, 18, 19, and 203 are hydrophobic and form a cluster on the surface of the enzyme, while in the cytosolic enzyme, residues 18 and 203 are hydrophilic. Substitution of a residue with noncomplimentary characteristics into this cluster of residues could destabilize the resulting enzyme structure. We could restore the stability of the enzymes by altering the surrounding residues to establish more favorable interactions. However, the wild-type mitochondrial native enzyme can tolerate the Y203H replacement, as shown in Table 3. Since residue 19 is on the surface of the subunit, it can be surmised that the orientation of residue 203 is important. Residue 203 and its nearest neighbors in the native mitochondrial isozyme are shown in Figure 3. The important interactions could be the hydrogen bond to Asp98 and hydrophobic interaction with Tyr101.

Allowing a favorable interaction to occur between residues 15 and 104 could also restore solubility but not activity (Table 3). Similar to the situation with residues 19 and 203, residues 15 and 104 do not make direct contact with the active site region and are located on the surface of the enzyme. Interestingly, the pairwise interactions were not reciprocal. Chimera 2 could only be made soluble when both residues 15 and 104 were those found in the cytosolic enzyme. This may be related to the altered main-chain conformation of residues 14 to 16 caused by the exchange of Pro for Leu at position 15 and the manner in which these residues interact with residue 104 and its immediate surroundings. Apparently, simple residue exchanges cannot establish the proper context for the interactions between residues 15 and 104. The restoration of solubility for this and for the 203 mutant must be related to both

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<th>Position of amino acid</th>
<th>15</th>
<th>104</th>
<th>18</th>
<th>19</th>
<th>203</th>
<th>Solubility*</th>
<th>Activity</th>
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<td>L</td>
<td>T</td>
<td>Q</td>
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<td>H</td>
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<td>Yes</td>
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<td>F</td>
<td>C</td>
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<td>Yes</td>
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<td>Yes</td>
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<td>F</td>
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<td>H</td>
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*The solubility was investigated by comparing ALDH protein in the supernatant and pellet from the E. coli extract.
the interactions of these residues with other residues more toward the interior of the enzyme, as well the manner in which the first few residues interact with the rest of the protein.

It has been reported that most chimeric proteins have reduced stability (Carriere et al., 1997; Mockus et al., 1997; Ahmad et al., 1998; Tasara et al., 1999). The data presented here show that surface amino acids that are not in contact with other subunits are important for maintaining the stability of the tetrameric ALDH. There appears to be one or two amino acids in these first 21 amino acids (residues 15 and 19), which happens not to be conserved, that make contact with another nonconserved residue (104 and 203, respectively). It is these interactions that are critical for maintaining the solubility of chimera 2, a predominant mitochondrial enzyme. In the native mitochondrial enzyme, though, it was possible to disrupt the interaction between residues 19 and 203 and still maintain a soluble enzyme. This means that this interaction alone is not critical for the stability of the native enzyme, but the chemical nature of these interactions, in context with the surrounding amino acid side chains that clearly influence the resulting overall stability of the final protein product when wholesale residue exchanges are made. The chimera made with the cytosolic enzyme fused to the first 21 amino acids of the mitochondrial enzyme was active, showing that if the protein is predominantly cytosolic the 19–203 interaction is of less importance. Though the cytosolic and mitochondrial isozyme share 70% sequence identity and have essentially identical three-dimensional structures, interactions between surface residues and interior ones are critical for maintaining the solubility of the enzymes. It is often implied in publications that a mutation or alteration to a surface residue is well tolerated by the protein since this residue will not disrupt hydrophobic packing of the molecule (Fushinobu et al., 1996). Here we show that in the case of the first 21 residues of ALDH, surface residues that are not conserved can affect the solubility of the enzyme. Each isozyme appeared to have evolved to possess pairs of amino acids that have the ability to stabilize the structure of that particular isozyme. The cytosolic form, though, seems to be more tolerant to change. In a subsequent publication, the stability of the two isozymes of ALDH will be discussed. It will be shown that the cytosolic isozyme is innately more stable than is the mitochondrial isozyme.

Materials and methods

Materials

NAD$^+$ was purchased from Sigma Chemical Company (St. Louis, Missouri); propionaldehyde was from Aldrich Chemical Company, Inc. (Milwaukee, Wisconsin); Sequenase Version 2.0 kit was obtained from U.S. Biochemical Corp. (Cleveland, Ohio); Magic Minipreps DNA purification system and T4 DNA ligase were from Promega Corp. (Madison, Wisconsin); alkaline phosphatase-conjugated goat anti-rabbit IgG and Muta-Gene in vitro mutagenesis kit were from Bio-Rad Laboratories, Inc. (Hercules, California); GeneClean kit was from Biol 101, Inc. (La Jolla, California); [$\alpha^35$S]dATP was from Amersham Corp. (Piscataway, New Jersey); the restriction enzymes and PCR reagents were from either New England Biolabs (Beverly, Massachusetts) or Promega Corp.; nitrocellulose membranes were from Schleicher and Schuell Inc. (Keene, New Hampshire).

Plasmid and bacterial strains

The cDNAs of human ALDH1, ALDH2, the 2 chimeras, and the chimera 2 mutants were cloned on pT7-7 expression vector, a derivative of pT7-1 (Tabor & Richardson, 1985), and expressed in Escherichia coli strain BL21 (DE3) pLysS (Studier & Moffatt, 1986) as previously reported (Zheng et al., 1993).

Construction of the chimeric cDNAs

All the chimeric cDNAs were constructed by splicing of overlap extension polymerase chain reaction (PCR) (SOE-PCR) (Ho et al., 1989; Horton et al., 1989; Yon & Fried, 1989). The PCR products were digested by Nde I and BamH I, and cloned into the same restriction sites of pT7-7 vector. The sequence of each chimeric cDNA was confirmed by double-stranded DNA sequencing of the pT7-7 plasmid (Sanger et al., 1977).

Oligonucleotide-directed mutagenesis

To construct the chimera 2 mutants, L15P, Q18F, Y19C, A104T, and Y203H, the oligonucleotide primers containing the mutation
Expression and purification of the chimeras

The cdNA of all of the chimera 2 mutants were transformed in BL21 (DE3) pLysS cells possessing chloramphenicol resistance pLysS plasmid. ALDH1, ALDH2, and all of the chimeras and mutants were expressed and purified by protamine sulfate treatment (1.25 mg/mL), DEAE-cellulose and 4-hydroxyacetophenone-based affinity chromatography (Jeng & Weiner, 1991; Ghent et al., 1992; Wang & Weiner, 1995). The purity of the enzymes was determined by SDS-polyacrylamide gel electrophoresis using the Coomassie Blue staining procedure. Fractions containing only chimeric ALDH were pooled and concentrated using a Centricon unit (Amicon, Beverly, Massachusetts). The pure enzyme was stored at −20 °C in the presence of 50% glycerol.

Fluorescence assay for the dehydrogenase activity

The dehydrogenase activity assays were performed by measuring the rate of increase in the fluorescence of NADH at 350–450 nm and 280 nm, respectively, at 25 °C (Farré et al., 1994). The $K_a$ and $V_{max}$ values for NAD$^+$ were determined in the presence of 14–140 μM propionaldehyde for different mutant forms of the enzyme, and the $K_a$ and $V_{max}$ values for propionaldehyde were determined in the presence of 1 mM NAD$^+$.

Crystallization and structure determination

Recombinant human mitochondrial aldehyde dehydrogenase was crystallized at 8 mg/mL from solutions containing 100 mM ACES, pH 6.2, 100 mM guanidine-HCl, 2 mM NAD$^+$, 3 mM diethio-itol, and 17% (w/v) PEG 6000. The crystals grew as flat plates and were soaked in 8 mM MnCl2 for two days prior to data collection. The crystals were flash-frozen using 30% ethylene glycol as the cryoprotectant prior to data collection at 113 K. A data set to 2.58 Å was collected with a crystal to detector distance of 180 mm and a 2 θ offset of 7.5° on a RAXIS IIC area detector. All the diffraction data were integrated, merged, and scaled with the HKL program package (Otwinowski & Minor, 1997). The structure of the human mitochondrial aldehyde dehydrogenase was solved by molecular replacement using the coordinates for the beef mitochondrial aldehyde dehydrogenase (1AG8) and the program AmoRe (Navaza, 1994). The molecular replacement solution was refined using the program X-PLOR (version 3.845, Brünger, 1988) and the data between 45 and 2.58 Å. The refinement included the application of a bulk solvent correction (Jiang & Brünger, 1994) and individual restrained temperature factors for all atoms. Tight eightfold non-crystallographic symmetry (NCS) restraints were maintained throughout the refinement process. Separate NCS restraints were applied to those cofactor molecules that exhibited similar conformations (three in one set, five in the other).

Determination of protein concentration

The protein concentration was determined with the Bio-Rad protein assay kit, using bovine serum albumin as a standard.

Acknowledgments

This work was supported by NIH Grants AA05812 (H.W.) and AA11982 (T.D.H.). This is journal paper number 16077 from the Purdue Agricultural Experimental Station. The coordinates and structure factors for the human ALDH2 structure have been deposited with the Protein Data Bank under the accession code 1CW3.

References


