Articles

Structural Aspects of Aldehyde Dehydrogenase that Influence Dimer−Tetramer Formation†

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ABSTRACT: Aldehyde dehydrogenases are isolated as dimers or tetramers but have essentially identical structures. The homotetramer (ALDH1 or ALDH2) is a dimer of dimers (A−B + C−D). In the tetrameric enzyme, Ser500 from subunit "D" interacts with Arg84, a conserved residue, from subunit "A". In the dimeric ALDH3 form, the interaction cannot exist. It has been proposed that the formation of the tetramer is prevented by the presence of a C-terminal tail in ALDH3 that is not present in ALDH1 or 2. To understand the forces that maintain the tetramer, deletion of the tail in ALDH3, addition of different tails in ALDH1, and mutations of different residues located in the dimer−dimer interface were made. Gel filtration of the recombinantly expressed enzymes demonstrated that no change in their oligomerization occurred. Urea denaturation showed a diminution to the stability of the ALDH1 mutants. The K_m for propionaldehyde was similar to that of the wild-type enzyme, but the K_m for NAD was altered. A double mutant of D80G and S82A produced an enzyme with the ability to form dimers and tetramers in a protein-concentration-dependent manner. Though stable, this dimeric form was inactive. The tetramer exhibited 10% activity compared with the wild type. Sequence alignment demonstrated that the hydrophobic surface area is increased in the tetrameric enzymes. The hydrophobic surface seems to be the main force that drives the formation of tetramers. The results indicated that residues 80 and 82 are involved in maintaining the tetramer after its assembly but the C-terminal extension contributes to the overall stability of the assembled protein.

Recently the structures of three forms of mammalian ALDH[1] were determined. Although the various proteins share less than 40% sequence identity (*1*), their three-dimensional structures were very similar (*2*−*5*). One striking difference in both sequence and structure is the C-terminal "tail". For a number of tetrameric ALDHs, the proteins terminate near the 500th residue. Those proteins that are known to be dimeric have an extension of amino acids that goes beyond the 500th residue but are lacking some residues found at the N-terminal of the tetrameric enzymes. For example, class 3 human stomach ALDH contain 453 amino acids compared to the class 1 or 2 human liver forms that have 500 amino acids each. The missing 59 residues all lie at the N-terminal end of the protein, a region that is on the surface and makes no contact with other subunits; hence, it might not play an important role in oligomerization. The homodimeric enzyme contains a pair of interacting subunits, while the tetrameric enzyme contains a pair of dimers. This C-terminal tail region is located in an area that could be considered to be where the pair of dimers in the tetrameric enzyme interact. Thus, it is possible that the C-terminal extension could interfere with tetramer formation.

It has been demonstrated that the N-termini of tetrameric ALDH is important for the stability (*6*) and folding (*7*) of the protein. In contrast, the C-terminal end of the protein extends through the dimer−dimer interface and could form an anchor between members of each dimer pair. This is illustrated in Figure 1a.

The three human ALDH isozymes have conserved amino acids involved in the substrate and coenzyme binding and catalysis, but they also have conserved amino acids that are not involved in the active site region, such as Arg84 (Arg25

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[1] Abbreviations: ALDH1, aldehyde dehydrogenase class 1; ALDH2, aldehyde dehydrogenase class 2; ALDH3, aldehyde dehydrogenase class 3; ALDH−5aa, aldehyde dehydrogenase class 1 with the addition of five amino acids at the C-terminus; ALDH−H3Tail, aldehyde dehydrogenase class 1 with the addition of the C-terminal tail of class 3; ALDH−GFP aldehyde dehydrogenase class 1 with the addition of the green fluorescent protein to the C-terminus; ALDH1−R84Q, aldehyde dehydrogenase class 1 with the mutation of arginine 84 to glutamine; ALDH1−D80G, aldehyde dehydrogenase class1 with the mutation of aspartic acid 80 to glycine; ALDH1−S82A, aldehyde dehydrogenase class 1 with the mutation of residue serine 82 to alanine; ALDH1−D80G/S82A, double mutant of ALDH1; ALDH1−R84G/D80G/S82A; triple mutant of ALDH1; ALDH3−Δtail, class 3 ALDH missing the C-terminal 17 amino acids; ALDH3−TailE436S, class 3 ALDH missing the 17 amino acid tail with a mutation of glutamic acid to serine as the C-terminal residue; SDS−PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate; GFP, green fluorescent protein.

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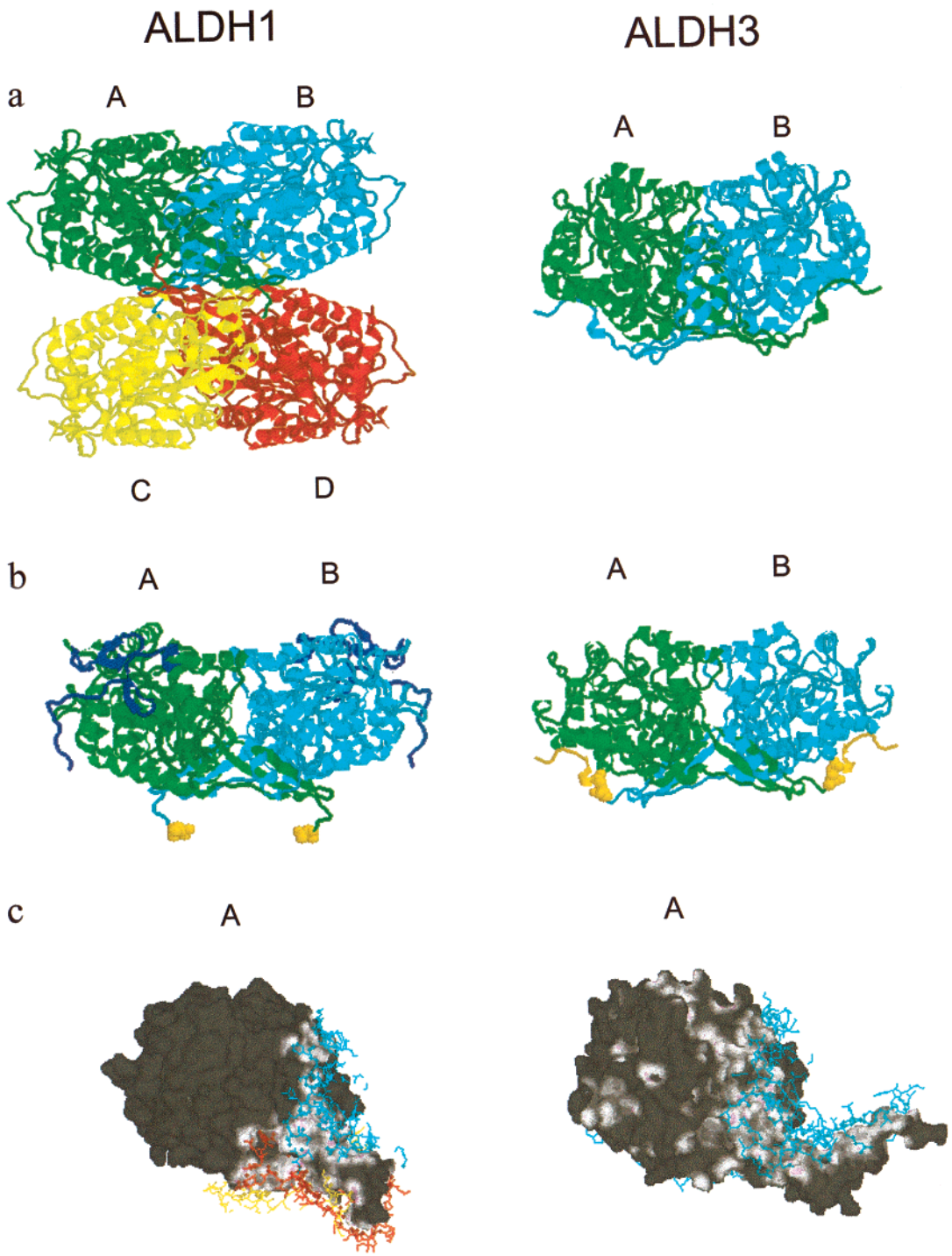

FIGURE 1: (a) Subunit arrangement in ALDH1 and ALDH3. (b) The N-terminus and C-terminus of ALDH1 and 3. In ALDH1 the N-terminus (purple) is located at the surface of the protein distal from the dimer−dimer interface, while the C-terminus goes through the dimer−dimer interface to interact with the other subunit. Class 3 ALDH begins what would be the corresponding 59th residue of the class 1 enzyme. Further, it extends 17 residues beyond where the class 1 enzyme terminates. The C-terminus portion of the protein is pointing away from what would be the dimer−dimer interface in the tetramer. Residues Ser500 in ALDH1 and Glu436 in ALDH3 are presented as space filled molecules. These residues are located in equivalent position in the proteins. (c) Residues within 4.0 Å from other subunits that are in contact with subunit A. Cyan is for the B subunit and yellow and red for the C and D subunits, respectively.

in class 3) (*8*). It has been proposed that the interaction of Arg84 with Ser500 from a monomer that is not part of the dimer pair is important for maintaining the tetrameric structure (*4*). This serine belongs to the monomer designed as "D" in the tetramer. A mutation of Arg84 in human liver mitochondrial ALDH2, produced an enzyme with 3-fold less activity, but without changes in the K_m for the substrates (*9*). Changes in quaternary structure, however, and stability were not determined. This Arg84−Ser500 interaction does not exist in the class 3 dimeric enzyme, but the Arg residue is still conserved. It interacts with Ser21 in its own subunit. An intersubunit interaction in the dimer pair is prevented by the 17-amino-acid tail at the C-termini (Figure 1b) (*10*).

The structural bases for some ALDHs being dimers and other being tetramers is not known. In addition to the C-terminal tail, the subunit contact area could be of

importance. In this study different strategies were utilized to evaluate the role of the tail, and some residues from the dimer−dimer interface, on the quaternary structure of two forms of ALDH.

MATERIALS AND METHODS

Expression System. Plasmids containing the cDNA clones of human ALDH3 and ALDH1 were used as the starting templates. Introduction of the mutations was performed by PCR using synthetic oligonucleotides as described (*6*, *9*, *11*). A polyhis tag at the N-terminus of the protein was introduced to facilitate purification. The resulting plasmids were transformed into the *Escherichia coli* BL21 strain for expression. Overnight cultures of transformed cells were used to inoculate 4 L of 2XYT medium. A subsequent incubation for 2.5−3 h (or $O.D._{600\ nm} = 0.5$) at 37 °C was conducted. Protein expression was induced by the addition of 0.4 mM isopropylthio-β-D-galactoside as described (*12*), and the incubation was continued overnight at 16 °C. The cells were harvested by centrifugation, washed twice with isotonic saline, and stored at −70 °C until used.

Protein Purification. Recombinant enzymes were first purified using a Chelating-Sepharose column charged with nickel acetate, as previously described (*13*). The column was washed with 2 volumes of buffer containing 50 mM sodium phosphate pH 7.5, 500 mM NaCl, 20 mM β-mercaptoethanol, and 20 mM imidazole; the enzymes were eluted from the column using a 0−500 mM imidazole gradient in the same buffer. Fractions containing the enzyme were identified by activity and SDS−PAGE gels (*14*). Enzyme fractions were pooled and dialyzed overnight against 2 L of buffer containing 50 mM sodium phosphate pH 7.5, 50 mM NaCl, 1 mM EDTA, and 0.1 mM dithiothreitol at 4 °C. Thereafter, ALDH3 and ALDH3−Δtail were loaded onto a Blue-Sepharose column. The enzyme was eluted with a salt gradient (0−500 mM NaCl). ALDH1, ALDH1−5aa, ALDH1−R84Q, ALDH1−D80G and ALDH1−S82A were purified using a hydroxyacetophenone-Sepharose affinity column (15). The column was washed several times with a buffer containing 20 mM sodium phosphate, pH 7.5, 0.2 mM dithiothreitol, 1 mM EDTA, and 50 mM NaCl and then eluted using 10 mM *p*-hydroxyacetophenone in the same buffer. ALDH1−H3Tail was purified using an AMP-Sepharose column, since this enzyme did not bind to the hydroxyacetophenone-Sepharose column; the enzyme was loaded onto the column and washed with 10 volumes of buffer containing 10 mM sodium phosphate, pH 7.4, and 0.2 mM dithiothreitol. Enzyme was eluted using 10 mM NAD in the above buffer. Pure enzymes were concentrated and stored in 50% glycerol at −20 °C until used.

Activity assays were performed by measuring the increase of fluorescence due to the NADH formation in a buffer containing 100 mM sodium phosphate, pH 7.5, and 100 mM NaCl at 25 °C (*9*).

Molecular Weight Analysis. Size exclusion analysis was performed using a Bio-Sil Sec-250 gel filtration column (Bio-Rad Laboratories). A 200 μL sample (0.5−1 mg/mL) was loaded onto the colum, and the elution of the protein was performed using a buffer containing 100 mM sodium phosphate, pH 7.4, 100 mM NaCl, 20 mM β-mercaptoethanol, and 10 mM NaN_3. Absorbance at 280 nm was measured and activity determined.

Urea Denaturation. Urea denaturation was determined by incubating the enzyme for 3 h at the desired urea concentration in assay buffer at 25 °C. The change in intrinsic fluorescence were measured using a Fluorolog-3 spectrofluorometer equipped with stirrer and temperature controller (ISA JOBIN YVON-SPEX Instruments S. A., Inc). The excitation wavelength used was 290 nm, and the emission spectrums were acquired from 300−400 nm.

Analytical Ultracentrifugation. Equilibrium centrifugations were performed on a Beckman XL-1 in 100 mM phosphate at pH 7.4 and 10 mM NaCl. Samples were spun at 6500 rpm for 20 h at 4 °C. Weight-average molecular weights were calculated using software provided National Analytical Biotechnology Center.

Surface Area. Calculations of the intersubunit contact areas, the amount of hydrophobic and hydrophilic residues, the presence of salt bridges, the hydrogen bonds, the distances, and the residue interactions between subunits were performed by the use of the Protein Explorer program (free software by Erick Martz).

RESULTS

C-Terminal Alterations. Expression of the Chimeric Proteins. Mutants of class 1 and 3 ALDHs were constructed so as to alter the tails that were located at the C-terminal end of the proteins. The various chimeras made are illustrated in Figure 2. A five-amino-acid extension was added to disrupt the salt bond between Arg84 and Ser500. A GFP form was made to determine if it was possible to fit a large extension of amino acids at the C-termini. The modified proteins were expressed in *E. coli* BL21 and purified to over 95% homogeneity, as judged by SDS−PAGE. The proteins were all soluble despite the changes made. The yield after purification of the ALDH mutants compared with wild type was 70% for ALDH1−GFP, 45% for ALDH1−H3Tail, and 27% for ALDH1−5aa. The yield after purification of the ALDH3 mutants was 31% for both ALDH3ΔTail and ALDH3ΔTail-E436S compared with ALDH3 wild type.

Size Exclusion Analysis of the ALDH-tail Mutants. Gel filtration was used to determine if there were major changes in the mass of the proteins possessing an altered C-terminus region. ALDH1 eluted from the size exclusion column at 7.7 min; both ALDH1−5aa and ALDH1−H3Tail eluted from the column at essentially the same time as did the wild type, 7.7 and 7.6 min, respectively. ALDH1−GFP eluted at 6.6 min. Purified ALDH3 was eluted at 9.7 min. The ALDH3 mutants showed a peak with a maximum corresponding to that of the wild-type enzyme, but these peaks had a small shoulder corresponding to a higher molecular weight component. When the elution times were plotted along with standards, it was noted that none of the ALDH proteins eluted at their expected times (data is presented in the Supporting Information, Figure 1). Although the enzymes' migrations were very unusual in this column, the differences between dimers and tetramers could be observed. These results indicated that the class 1 mutants were still tetramers and the class 3 mutants were still dimers. Even with the addition of the GFP protein to the C-terminal end of the class 1, a tetramer was formed.

Both the class 3 mutants that were missing the C-terminal tail and the mutant where a change to the last amino acid

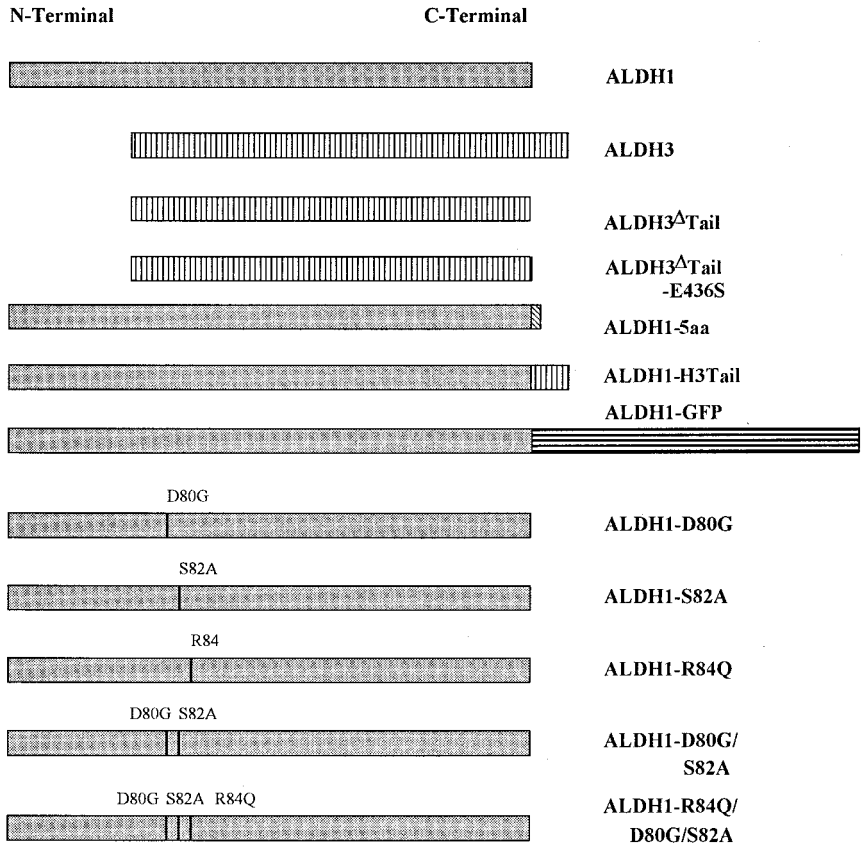

FIGURE 2: Representation of the ALDH constructs used in this study. In this diagram, the different tails added or deleted from the ALDH proteins and the sites for the point mutations are indicated.

Table 1: Kinetic Parameters of ALDH−Tail Mutants

enzyme	V_{max} (nmol min^{-1} mg^{-1})	K_m propionaldehyde (μM)	K_m NAD (μM)	K_{ia} (μM)
ALDH1	200	12	11	34
ALDH1−5AA	77	8	8	21
ALDH1−H3Tail	140	31	22	31
ALDH1−GFP	3	N.D.[a]	N.D.	N.D.

[a] Not determined.

Table 2: Kinetic Parameters of ALDH3 Mutants

enzyme	V_{max} (nmol min^{-1} mg^{-1})	K_m benzaldehyde (μM)	K_m NAD (μM)	K_{ia} (μM)
ALDH3	30	180	4	7
ALDH3−ΔTail	24	650	360	510
ALDH3−ΔTailE436S	28	320	1300	1800

was made showed some tendency toward having a higher molecular weight component present. There was, however, no evidence for the formation of tetrameric enzyme forms. Neither deleting the tail of the class 3 enzyme nor altering it on the class 1 enzyme caused the proteins to change their quaternary structure.

Activity of the ALDH Modified Proteins. The activity of the ALDH1 mutants decreased 30% and 60% for ALDH1−H3tail and ALDH1−5aa, respectively, compared with ALDH1 (Table 1). ALDH1−GFP mutant had less than 2% of the activity of the wild-type enzyme. The K_m values for propionaldehyde and NAD were unchanged for ALDH1−5aa and increased about 3 and 2 times, respectively, for ALDH1−H3Tail, while the K_{ia} was wild-type-like for both chimeric enzymes (Table 1).

The specific activity of the mutant class 3 enzymes was essentially unchanged from that of the native enzyme when NAD was the cofactor. The modification to the C-terminal tail, though, did affect NAD binding (Table 2), as both the K_m for NAD and K_{ia} values increased dramatically for the mutants. The K_m for aldehyde, in contrast, just increased two to 4-fold. Class 3 ALDH can use NADP nearly as well as NAD. The specific activity with NADP was 80% compared with the activity with NAD (data not shown). For the mutants, no activity was found with NADP. Apparently, the subtle change in structure caused by the change in the C-terminal tail affected the coenzyme binding domain.

Stability of the Modified Proteins Against Urea Denaturation. The effect of urea on the intrinsic fluorescence of the enzyme was measured to determine if there was a change in stability of the chimeric enzymes compared to the native enzyme. The intrinsic fluorescence of ALDH1−5aa and ALDH1−H3Tail were similar to that of ALDH1 in that each had a maximum emission at 340 nm in the absence of urea and a shift of the maximum to the region of 360−370 nm in the presence of urea (supplement Figure 2). ALDH1 was the most stable form of ALDH, requiring 3.7 M urea to reach 50% of the F_{app} (Figure 3A). The concentration of urea needed to obtain 50% of the apparent fluorescence for ALDH1−H3Tail and ALDH1−5aa was 1.7 and 1.4 M, respectively. ALDH1−5aa and ALDH1−H3Tail showed a urea denaturation curve that was similar to that of ALDH3. Though making changes to the C-terminal tail of ALDH1 reduced the stability of the proteins, it did not cause the enzymes to become dimers.

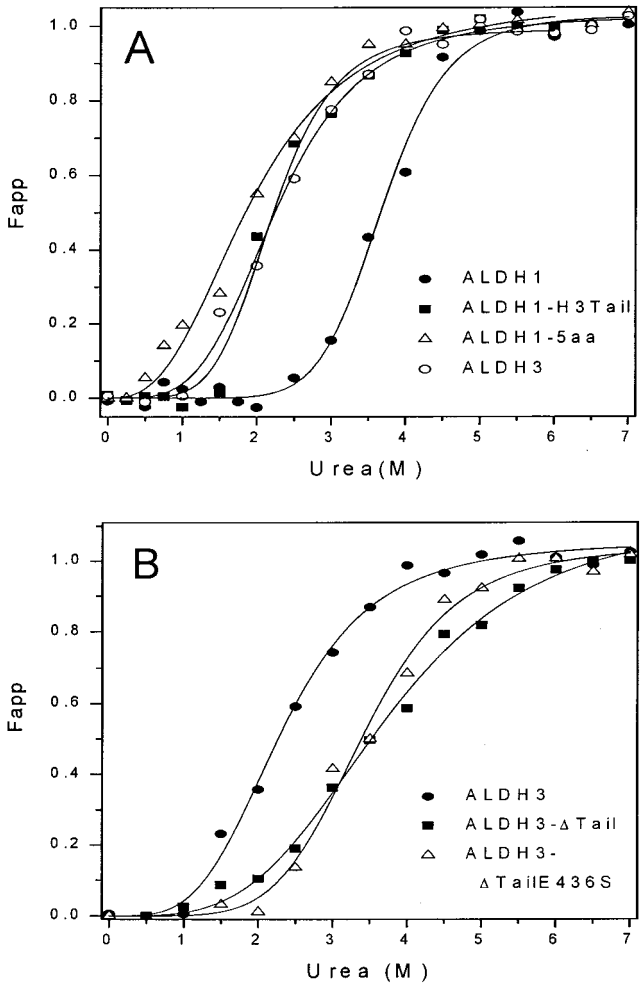

FIGURE 3: Urea denaturation of ALDH1 and ALDH3 mutants. The maximum of fluorescence in the presence (F_u) and the absence (F_c) of urea were obtained after 3 h of incubation at room temperature. These values were used to calculate the F_{app} for each point using the equation $F_{app} = (F_c - F)/(F_c - F_u)$. (A) The profiles from the ALDH1−tail mutants are less stable than those of the parent enzyme and similar to the profile from class 3. (B) The mutants generated from ALDH3 were more stable than those from the wild-type enzyme; their profile of denaturation resembled that from ALDH1.

Unlike with the class 1 mutants, the class 3 mutants all proved to be more stable than their parent enzyme (Figure 3B). This was an unexpected result since the binding for coenzyme changed so much with the class 3 truncated mutants indicating that some structural alterations occurred. Even though the mutant proteins remained dimeric their binding properties and stability changed.

Mutation of Residues in the Dimer−Dimer Interface in ALDH1. Cloning and Expression. Using the data from the crystal structure of ALDH1 and the computer program Protein Explorer, we determined the contact area between subunits (Figure 1C). Subunit A makes the most contact with subunit B (the subunit in the same dimer pair); it has very little contact with subunit D, and almost no contact exists with subunit C (*4*, *6*). The A/B−C/D dimer−dimer contact area involves 102 amino acids within 4.0 Å from each dimer pair. The dimer−dimer interface involves 60 hydrogen bonds, 34 hydrophobic interactions and 16 salt bridges. Assuming that buried salt bonds were important interactions that participated in maintaining the tetramer, some of the residues involved in these were mutated. The changes made were

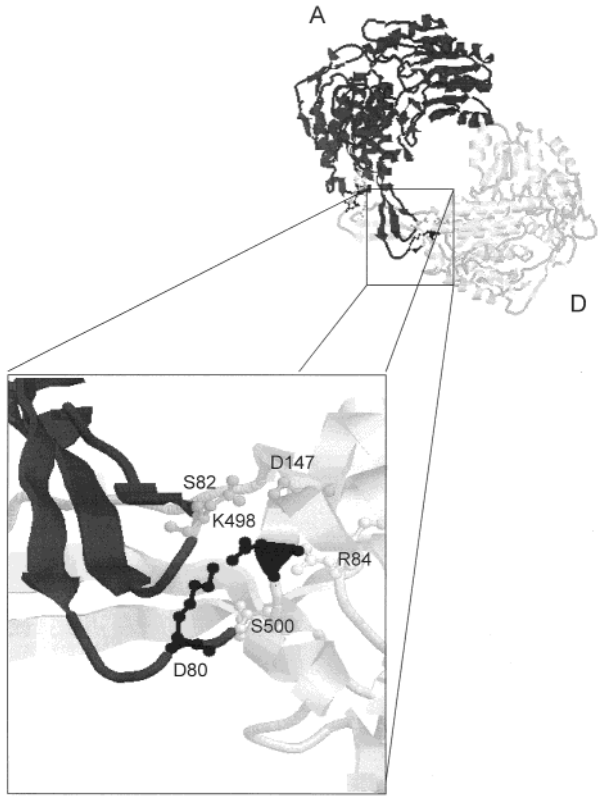

FIGURE 4: Ribbon presentation of the A and D subunits in ALDH1. A portion of the contact area between the A and D subunits is amplified for a better visualization of the residues. Residues that were mutated and their corresponding interacting amino acids are presented as ball + stick.

residues D80G, S82A, and R84Q, a combination of D80G and S82A to generate a double mutant, and a combination of the three residues to obtain a triple mutant. The residues that were mutated are illustrated in Figure 4. The mutants were all expressed in *E. coli* BL21 and purified as indicated under the Materials and Methods section. The yield after the purification of these proteins was 95%, 50%, and 20% for ALDH1−S82A, ALDH1−R84Q, and ALDH1−D80G, respectively, showing that the mutant might be less stable than the parent proteins. The triple mutant was essentially insoluble after expression, but the double mutant was stable; its yield after the purification was 20% of the yield of the wild-type enzyme.

Size Exclusion Analysis of the Intersubunit Mutants. Gel filtration analysis of the proteins possessing a single mutation showed that they remained as tetramers; their profiles overlapped with the profile of the native enzyme. The double mutant, however, showed two overlapping peaks on the gel filtration column. The first peak corresponded to the peak of the wild type, while the second one migrated in the region expected for a protein with a mass of 100 KD. The calibration curve and the elution times are shown in the Supporting Information as Figure 3. This result suggested that the D80G/S82A double mutant was a mix of dimers and tetramers. These results were corroborated by preliminary ultra centrifugation analysis. A weight-average molecular weight for the native enzyme was 220 000, consistent with that of a tetramer. For the double mutant, the value was 150 kDa, indicating that a mixture of dimers and tetramers existed.

Activity of the Mutant Enzymes. The activity of the three mutant enzymes, ALDH1−D80G, ALDH1−S82A, and

Table 3: Kinetic Parameters of ALDH1 Interface Mutants

enzyme	V_{max} (nmol min^{-1} mg^{-1})	K_m propionaldehyde (μM)	K_m NAD (μM)	K_{ia} (μM)	K_{iq} (μM)
ALDH1	200	12	11	34	124
ALDH1−R84Q	139	70	4	10	89
ALDH1−D80G	204	2.8	3.5	6	127
ALDH−S82A	123	4.4	1.7	2.3	43
ALDH1−D80G/S82A	15	N.D.[a]	N.D.	N.D.	N.D.

[a] Not determined.

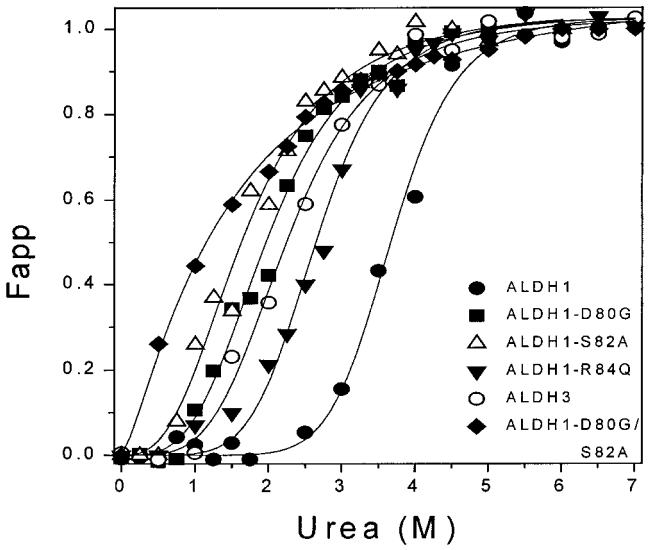

FIGURE 5: Urea denaturation of the point mutants of ALDH1. The stability of ALDH1−R84Q was midway between ALDH1 and ALDH3, while ALDH1−D80G and ALDH1−S82A presented stability profiles similar to that of ALDH3. The ALDH1−D80G/S82A double mutant was the least stable. Spectrums were acquired after 3 h of incubation at room temperature. The F_{app} was determined as indicated in Figure 3.

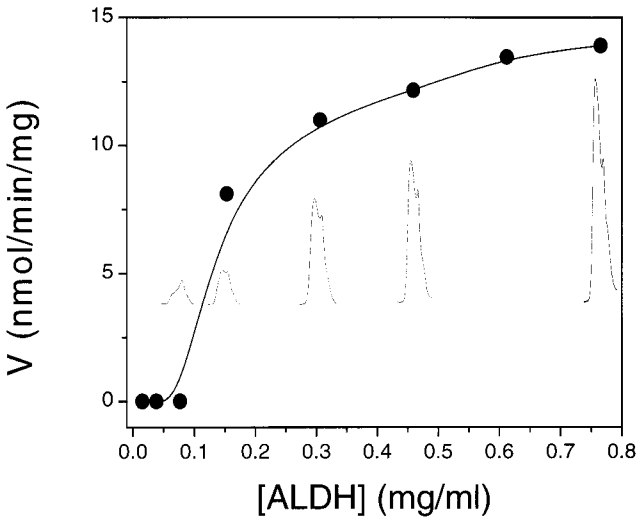

FIGURE 6: Effect of the protein concentration on the activity of the ALDH1−D80G/S82A double mutant. The elution profiles found at different enzyme concentrations are superimposed with the activity plot. The elution time was 7.5 min for the first peak and 8.3 min for the lower molecular weight component. When the species was primarily the dimer, the enzyme was inactive, while when the tetramer was present, activity was found. The highest specific activity of the mutants was 10% of the wild-type enzyme.

ALDH1−R84Q differed from each other. The first had activity like the native enzyme, while the S82A and the R84Q mutants had 60% and 70% activity, respectively, compared with native ALDH1. The K_m for propionaldehyde decreased 4- and 3-fold and increased about 6-fold for these enzymes, respectively. The K_m for NAD decreased 3 times for ALDH1−D80G and ALDH1−R84Q and 6.5 times for ALDH1−S82A. The K_{ia} decreased 5.7 times for D80G, 15 times for S82A, and 3 times for R84Q. K_{iq} changed by less than a factor of 2 for some mutants. The D80G/S82A mutant showed 7.5% of the activity of the wild type (Table 3).

Stability of the Intersubunit Mutants. The single mutants were less stable against urea denaturation than was the native enzyme. D80G and S82A showed stability resembling that of ALDH3, while R84Q was more stable. The urea concentration necessary to denature this mutant was midway between that necessary to denature ALDH1 and ALDH3 (Figure 5). The double mutant needed 0.6 M urea to reach 50% of the apparent fluorescence showing that this mutant was the most unstable. It was not possible to investigate the triple mutant because of its low solubility and stability.

Not only was the double mutant of class 1 ALDH less stable than the parent enzyme, but, as might be expected, it was denatured at a faster rate in the presence of urea. The fluorescence emission spectra of the proteins as a function of time showed that while the half-life for the denaturation of ALDH1 and the R84Q single mutant in 7 M urea was around 10 min, it decreased to less than 2 min for the D80G/S82A double mutant (Supplement Figure 4).

Protein Concentration Effects on the Oligomerization and on the Activity of ALDH1−D80G/S82A Double Mutant. When the double mutant was analyzed by gel filtration, two peaks were observed, indicating that it could be a mixture of tetramers and dimers. Performing the experiment at different protein concentrations showed that one peak diminished and the other increased in size. This result indicated that the dimer−tetramer formation in ALDH1−D80G/S82A was in equilibrium (Figure 6). This effect was not observed with the other mutants nor with the native enzyme. Initially, it was thought that the double mutant was inactive, but when assayed at a concentration of protein that made it appear that most of the enzyme was in the tetrameric form, it was found that the enzyme possessed 10% the specific activity of the parent tetrameric enzyme (Figure 6), consistent with what was shown in Table 3.

Estimation of the Stability of the Mutant Forms of ALDH. It was not possible to directly determine the changes in the stability of the various ALDH mutants used in this study since the urea denaturation was irreversible. It was, though, possible to make a relative comparison of the changes by analyzing the data at a fixed concentration of urea. It appeared that at 2 M urea all of the proteins were present as a mixture of the native and an altered state. It is not known if the altered state is an unfolded denatured state or a partially

Table 4: Relative Changes in the Stability of the Altered Forms of ALDH from Analysis of the Fluorescent Data in the Presence of 2M Urea

enzyme	ΔG°_{app} [a]	$\Delta\Delta G^{\circ}_{app}$ [b]
ALDH1	−7.8	−
ALDH1−H3Tail	−1.8	6.0
ALDH1−5aa	+1.0	8.8
ALDH3	−1.4	6.4
ALDH3−ΔTail	−7.1	0.7
ALDH3−ΔTailE436S	−9.7	−1.8
ALDH1−R84Q	−4.9	2.9
ALDH1−D80G	+0.4	8.2
ALDH1−S82A	+2.1	9.9
ALDH1−D80G,S82A	+3.0	10.8

[a] Assumes that two species are present and their concentrations can be estimated by the fluorescence values showed in Figures 3 and 5. The ΔG°_{app} is not a true measure of the stability of the enzymes in urea. [b] Difference in stability compared to ALDH1.

unfolded state. For an approximation of the changes in free energy caused by the alterations made to ALDH, it was assumed that there were just two species present in solution and that one was represented by the initial fluorescence value and the other by the final value. Thus, it was possible to assign a K^{eq}_{app} and hence a $\Delta\Delta G^{\circ}_{app}$ for each change made. These are tabulated in Table 4. The absolute value of $\Delta\Delta G^{\circ}_{app}$ has no physical meaning.

DISCUSSION

A large number of ALDHs have been identified and many characterized. Only a few amino acid residues are common among the entire super family of enzymes; however, the mammalian ones share around 20% sequence identity when the class 3 is compared to the class 1 enzyme but 70% when class 2 is compared to class 1 (*1*). The class 1 cytosolic and class 2 mitochondrial liver forms are among the best studied enzymes since their structures have been determined. Both are tetrameric and contain 500 amino acid residues in each subunit. This is in contrast to the other well-studied rat class 3 form. It is a dimer with 457 residues (*3*). On the basis of their three-dimensional structures, it appears that the "tail" at the C-termini could affect subunit assembly. It is located in what was considered to be the oligomerization domain (*4*). In contrast, the N-terminal portion appears to lie on the surface making no contact with other subunits. These structural features were illustrated in Figure 1.

It was unexpected to have found that state of assembly was relatively independent of the changes that were made to the C-terminal tail. It was neither possible to convert the dimeric form into a tetramer by removing the tail nor to convert a tetrameric form to a dimer by adding the tail. The overall stability of the protein, however, was greatly affected by the alterations. Denaturation of the class 1 enzyme occurred at a lower concentration of urea when the 17 amino acids of the class 3 enzyme were placed on it. In contrast, when the tail was removed from the class 3 dimeric enzyme, the stability against urea denaturation increased. From the $\Delta\Delta G^{\circ}_{app}$ values listed in Table 4, it can be estimated that adding the tail to the class 1 enzyme decreases stability by approximately 6 kcal/mol while removing it from the class 3 enzyme increased stability by perhaps 2 kcal/mol.

The kinetic properties of the class 1 enzyme were not drastically altered by changes made to the C-termini. In contrast, the class 3 enzyme lost its ability to use NADP as the coenzyme and had dramatic increases in K_m and K_{ia} for NAD. Apparently the loss of the interactions made by the C-terminal amino acids affected the coenzyme binding region especially near residues Glu140 and Lys137. These two residues are located near where the 2′ hydroxyl of the ribose in NADP would be in contact with the enzyme (*16*).

A salt bond exists between residues 84 and 500 in the tetrameric enzyme. The conserved arginine at position 84 makes a bond not with the subunit in the dimer pair, but across subunits, so there is a bond between the A and the D subunits. It was previously shown that making an R84Q mutant of the class 2 mitochondrial tetrameric enzyme caused the specific activity to decrease (*9*). Here we show that with the class 1 enzyme a similar diminution of activity occurred when the R84Q mutant was analyzed. Though the stability of this mutant decreased by approximately 3 kcal/mol compared to the native enzyme, it proved to be more stable than any other class 1 mutant used in this study. Thus, this conserved residue is not completely essential for the maintenance of the tetramer. This residue is present in the dimeric enzyme but obviously cannot be involved in the interaction as it is in the tetrameric enzyme. Rather than form a salt bond with the C-terminal carboxyl residue, it binds to Ser21 in its own subunit. Unfortunately, a corresponding mutation to the class 3 enzyme caused it to become insoluble. Hence, we could not determine the effect of breaking this bond on stability other than to observe that it was necessary to be present to have a soluble enzyme.

Along with Arg84, two other nearby nonconserved residues interact with the C-termini of a subunit in the tetramer. These are Asp80, forming part of a small loop, and Ser82, part of a helix (*4*, *5*). These residues interact with Lys498 and Asp147, respectively, from a monomer of the other dimer (*4*). Disruption of these interactions decreased the stability of the tetramer by 8 or 9 kcal/mol but did not affect its ability to be assembled after it was recombinantly expressed. It appears that the disruption of both the Asp80 and the Ser82 interactions could allow the double mutant protein to actually dissociate into dimers in a concentration dependent manner. This is consistent with a system in equilibrium. When activity was assayed at low concentrations of enzyme where dimers primarily existed, no catalytic activity was found unlike that found at a high concentration of protein. Why the enzyme was not active in the dimeric form is not understood, since each subunit appears from the structure to have its own active site (*4*).

Previous work from our laboratory showed that the horse liver mitochondrial ALDH could dissociate to a pair of dimers in the presence of Mg^{2+} ions (*17*). Neither we nor others have found evidence for the dissociation of any other ALDH. We cannot offer an explanation for the why the horse enzyme was found to dissociate. We could, though, investigate the ramification of having the human class 1 enzyme dissociate. In Figure 1, it was shown how one subunit interacts with its binding partner in the dimer as well as with the other dimer pair in the tetramer. Assuming the dimer pairs dissociated, one could look at the face that was between the dimers that now would be exposed to the buffer. If no major conformational changed occurred ,a large hydrophobic area would be exposed, as illustrated in Figure 7. Also shown in the Figure is the corresponding region of the class 3

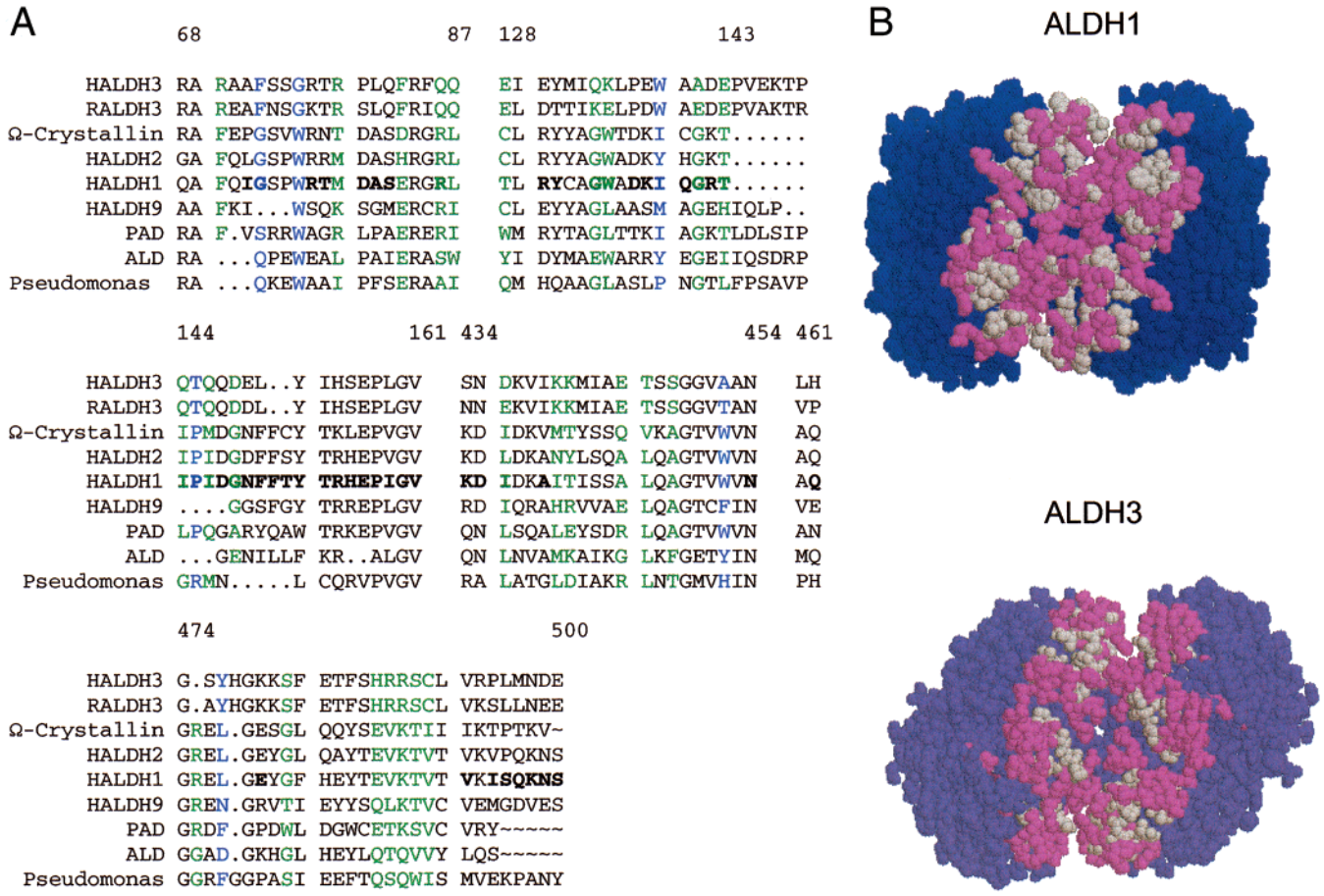

FIGURE 7: (A) Alignment of the amino acid sequence of the A−B, C−D dimer−dimer contact area of ALDH1, compared with the equivalent region of different ALDHs. The alignment showed that several residues of this region that are hydrophobic in the tetrameric enzymes are hydrophilic in the dimeric ones (residues in green). In this alignment, it can also be observed that some small residues in ALDH3 are changed to voluminous ones in ALDH1 (residues in blue). HALDH3, human stomach ALDH3; RALDH3, rat liver ALDH3; Ω-crystallin, ALDH from scallop lens; ALDH1, human liver cytosolic aldehyde dehydrogenase; ALDH2, human liver mitochondrial aldehyde dehydrogenase; ALDH9, human betaine dehydrogenase; PAD, phenyl acetaldehyde dehydrogenase from *E. coli*. ALD, aldehyde dehydrogenase from *E. coli*; Pseudomonas, benzaldehyde dehydrogenase from *Pseudomonas putida*. (B) Comparison of the amount of hydrophobic and hydrophilic residues in the dimer−dimer contact area of ALDH1 and the equivalent region on ALDH3. Hydrophobic residues are shown in light gray, and hydrophilic residues are shown in purple. In this area, hydrophobic residues are increased in ALDH1 with respect to ALDH3.

dimeric protein. It can be noted that the region in the class 3 enzyme is much more hydrophilic. A comparison of the amino acids found in this region of the tetrameric enzyme area is also presented in Figure 7. The dimeric forms have less hydrophobicity, as would be expected for a surface that was exposed to the buffer.

The sequence of Ω-Crystallin is similar to the class 1 and 2 enzymes, but this protein was reported to be a dimer. Although the enzyme possesses all the residues necessary for the activity, it was found that the enzyme was inactive (*18*). This protein was purified under denaturation conditions and then renatured. The methods used to purify the protein could be the cause for the formation of a dimer and the reason no activity could be detected.

It is impossible to explain the evolutionary pressure that would cause some forms of ALDH to remain dimeric while others became a dimer of dimers forming a tetrameric enzyme. The additional amino acids at the C-terminal end, though a good predictor of whether an ALDH was dimeric, does not seem to be the governing feature that allows or prevents assembly. The driving force appears to be more related to surface area that would be exposed if a tetramer became a pair of dimers.

Dramatic alterations to the C-terminal end of the ALDHs affected the kinetic properties of the enzyme. The most startling observation was that the class 3 enzyme lost its ability to use NADP as a coenzyme and increased the K_m for NAD when the tail was removed. Adding a tail to the tetrameric form did not greatly affect its kinetic properties even though the stability was affected.

Previously, it was reported that tetrameric aldolase A could become either active dimers (*19*) or monomers (*20*) by mutating residues on the different contact surfaces of the tetramer. Another report showed the formation of dimers for the normal tetrameric phosphorylating glyceraldehyde 3-phosphate dehydrogenase, a member of the ALDH family (*21*). The strategy in this work was again site-directed mutagenesis of residues located on the dimer−dimer interface. Similar to our observations, the resulting dimers of this enzyme were inactive, but they were still able to bind NAD. Though the region where the mutations were made in the ALDHs was distal from the active site, the resulting dimers were inactive. These results seem to indicate that the oligomerization is not related to the activity in aldolase A but is in ALDH1 and glyceraldehyde 3-phosphate dehydrogenase. The fact that the double mutant of class 1 ALDH was still able to form

tetramers indicated that factors other than the intersubunit bonding were participating in the assembly of dimers. Alignment of different ALDHs known to be dimers or tetramer showed that in the dimer−dimer contact region, the hydrophobic surface is increased in the tetramer compared with the equivalent region in the dimeric proteins. In the dimeric class 3 ALDH, the side chains of surface hydrophobic amino acids are pointing to the interior of the protein and not to surface (*3*). The forces that drive assemble of the dimers appears to be the hydrophobic surface in the dimer−dimer contact area, and once assembled, salt bonding maintains the tetramer. The surface exposed when either aldolase or glyeraldehyde-3-phosphate dehydrogenase dissociates appear to be much more hydrophilic than that of class 1 ALDH. Thus, those two proteins remain soluble as dimers. It is concluded that the C-terminal end of the ALDHs are involved in the ultimate stability of the protein, but an important factor in their being a dimer or a tetramer is the surface that would be exposed to buffer if they were in a different state of assembly.

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SUPPORTING INFORMATION AVAILABLE

Four figures are presented. Figure 1 shows the elution of the ALDH3 mutants from the size exclusion column and a molecular weight calibration curve for both the mutants of ALDH3 and ALDH1. Figure 2 shows the fluorescence emission curves as a function of time for class1 ALDH tail-mutants in the presence of 7M urea. Figure 3 shows the elution of the double mutant of ALDH1 from the size exclusion column and a molecular weight calibration curve. Figure 4 shows the fluorescence emission curves as a function of time for class1 ALDH point-mutants in the presence of 7M urea. This material is available free of charge via the Internet at http://pubs/acs.org.

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