Cooperativity in Nicotinamide Adenine Dinucleotide Binding Induced by Mutations of Arginine 475 Located at the Subunit Interface in the Human Liver Mitochondrial Class 2 Aldehyde Dehydrogenase†

Baoxian Wei,‡ Li Ni,‡ Thomas D. Hurley,§ and Henry Weiner*,‡

Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153, and Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202-5122

Received January 6, 2000; Revised Manuscript Received February 24, 2000

ABSTRACT: The low-activity Oriental variant of human mitochondrial aldehyde dehydrogenase possesses a lysine rather than a glutamate at residue 487 in the 500 amino acid homotetrameric enzyme. The glutamate at position 487 formed two salt bonds, one to an arginine at position 264 in the same subunit and the other to arginine 475 in a different subunit [Steinmetz, C. G., Xie, P.-G.,Weiner, H., and Hurley, T. D. (1997) *Structure 5*, 2487−2505]. Mutating arginine 264 to glutamine produced a recombinantly expressed enzyme with nativelike properties; in contrast, mutating arginine 475 to glutamine produced an enzyme that exhibited positive cooperativity in NAD binding. The K_M for NAD increased 23-fold with a Hill coefficient of 1.8. The binding of both NAD and NADH was affected by the mutation at position 475. Restoring the salt bonds between residues 487 and either or both 264 and 475 did not restore nativelike properties to the Oriental variant. Further, the R475Q mutant was thermally less stable than the native enzyme, Oriental variant, or other mutants. The presence of NAD restored nativelike stability to the mutant. It is concluded that movement of arginine 475 disrupted salt bonds between it and residues other than the one at 487, which caused the apo-R475Q mutant to have properties typical of an enzyme that exhibits positive cooperativity in substrate binding. Breaking the salt bond between glutamate 487 in the Oriental variant and the two arginine residues cannot be the only reason that this enzyme has altered catalytic properties.

Acetaldehyde, formed during ethanol oxidation, is primarily oxidized by an isozyme of liver aldehyde dehydrogenase that is located in the matrix space of the mitochondria *(1)*. Many Asian people possess a null mutant of the enzyme where a lysine replaces the glutamate at position 487 in the 500 amino acid homotetrameric enzyme. These people "flush" when they consume alcoholic beverages *(2, 3)* due to decreased metabolism of the acetaldehyde produced from ethanol.

Many properties of the Oriental variant of ALDH[1] were known prior to the determination of the structure of the active mitochondrial isozyme. It was shown that though the initial reports suggested that the E487K enzyme was inactive *(4)*, the Oriental variant actually possessed some catalytic activity. The specific activity was just 10% that of the native enzyme, but the K_M for NAD increased 200-fold *(5)*. A glutamine substitution was not detrimental in that the E487Q had nativelike K_M and V_{max} values. Thus, it was not the loss of the glutamate at position 487 that caused the Oriental variant to have such altered properties when compared to the native enzyme. Instead, it was the introduction of the positive charge. Before knowing the structure of the liver mitochondrial enzyme, we hypothesized that residue 487 might reside near the nicotinamide ring. The fact that the Oriental variant had an increased K_{ia} for NAD but not for NADH supported the supposition since the nicotinamide ring of NAD would be positive, while that of NADH would not be.

After the structure of the beef liver mitochondrial isozyme *(6)* and later that of the human form was determined *(7)*, it became apparent that residue 487 was not located near the nicotinamide ring. Instead, it was found to be in close proximity to two arginine residues at the interface between subunits. One of these arginine residues, at position 264, was located in the same subunit while the other, Arg475, was in the subunit that comprised the dimer pair in the pair of dimers. It was of interest for us to find that glutamate 487 was in contact with residues in two different subunits for we knew that in a heterotetramer, the Oriental subunit was dominant and affected the activity of both subunits *(8)*.

It appeared that the major cause for the reduced activity of the Oriental variant of ALDH could be related to the disruption of the salt bonds between the glutamate and arginine residues. To test concept, mutations were made to

† This research was supported by NIH Grants AA05812 (H.W.) and P60-AA07611(T.D.H.). This is paper no. 16261 from Purdue University Agriculture Experiment Station.

* To whom correspondence should be addressed: Telephone (765) 494-1650; Fax: (765) 494-7897; Email Weiner@biochem.purdue.edu.

‡ Department of Biochemistry, Purdue University.

§ Department of Biochemistry and Molecular Biology, Indiana University.

[1] Abbreviations: ALDH, aldehyde dehydrogenase; Oriental variant, the form of aldehyde dehydrogenase found in many Asian people that has a lysine at position 487 rather than a glutamate; NPA, *p*-nitrophenyl acetate; SDS−PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate; R475X, arginine 475 mutated to another residue.

10.1021/bi000028n CCC: $19.00
Published on Web 04/13/2000

arginine residues 264 and 475 of the human mitochondrial enzyme. Unexpectedly, mutations of arginine 475 caused the enzyme to become one where cooperativity in NAD binding occurred, as will be shown in this study. It is possible that an alteration of the environment of this arginine residue is the actual cause of the changed properties found in the Oriental variant of ALDH.

MATERIAL AND METHODS

Material. NAD and NADH were purchased from Sigma; propionaldehyde, chloroacetaldehyde, and *p*-nitrophenyl acetate were from Aldrich. SequiTherm Excel II DNA sequence kit was purchased from Epicenter Technologies, Inc; restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs or Promega Corp. Qiaspin miniprep plasmid purification and Qiaex II gel extraction kits were from Qiagen. Alkaline phosphatase-conjugated goat anti-rabbit IgG was from Bio-Rad laboratories. [α-^{35}S]dATP was purchased from Amersham Corp. Synthetic oligonucleotides used for seqencing and mutagenesis were purchased from Integrated DNA Technologies, Inc.

Cells and Plasmid. Native and mutant ALDH cDNAs were cloned into a pT7-7 expression vector, which is a derivative of pT7-1 described by Tabor and Rochardson *(9)* and transformed into the *E. coli strain* BL21 (DE3) (pLysS) *(10).* Protein expression was conducted as previously described *(11).*

Mutagenesis of ALDH. Single mutations were introduced into the ALDH gene by use of synthetic oligonucleotides and polymerase chain reaction techniques previously described *(7, 8, 12).* The mutants were confirmed by double-stranded DNA sequencing with a thermocycler sequencing kit. Double and triple mutants were constructed by exchanging the cDNA fragments containing the single mutants with the corresponding fragments of the native ALDH or E487K cDNA from the pT7-7 plasmid. All mutations were again confirmed by double-strand DNA sequencing of the pT7-7 plasmid.

Purification of Native and Mutant Enzymes. Recombinantly expressed native or mutant enzymes were purified through DEAE-SP (Sigma) and *p*-hydroxyacetophenone affinity chromatography *(13)* as previously described *(14).* During purification, the activity of the recombinantly expressed ALDHs was monitored at pH 7.4. For the Oriental mutant enzymes, dot blotting was used to determine the fractions containing mutant enzymes. Final enzyme purity (estimated to be >95%) was assessed by Coomassie Blue-stained SDS−PAGE *(15).* The final protein concentration was determined with a Bio-Rad protein assay kit with bovine serum albumin as a standard. The purified enzymes were concentrated with an Amicon stirred-cell concentrator and stored at −20 °C in the presence of 50% glycerol.

Fluorescence Assay for the Dehydrogenase Activity. Dehydrogenase activity assays were performed by measuring the rate of increase in the fluorescence of NADH formation in 100 mM sodium phosphate (pH 7.4) at 25 °C *(5)* with an Aminco filter fluorometer. Reactions were initiated by the addition of propionaldehyde to buffer containing enzyme and NAD, and the change in fluorescence was recorded as a function of time. The standard assay contained 1 mM NAD and 14 μM propionaldehyde.

Spectroscopic Assay for the Esterase Activity. Activity assays were performed by measuring the hydrolysis of *p*-nitrophenyl acetate at 400 nm in 100 mM sodium phosphate (pH 7.4) at 25 °C in the absence or presence of added NAD or NADH *(16).*

Enzyme Kinetics. All kinetic measurements were performed at least three times, and the mean values were used for calculations or plots. Kinetic parameters were obtained from the MicroMath Scientist computer program.

Pre-Steady-State Burst of NADH Formation. The pre-steady-state burst magnitude of NADH formation was determined with a Hitachi 2000 fluorescence spectrophotometer as previously described *(17).* Enzyme and NAD were incubated in 100 mM sodium phosphate (pH 7.4) to establish a fluorescence baseline. At time zero, propionaldehyde (140 μM) was added to initiate the reaction. The extrapolated line intersection at time zero gave the magnitude of the burst of NADH formation. Concentrations of NAD were 1 and 10 mM for the native and R475Q mutant enzymes, respectively.

Thermal Stability. The native and mutant enzyme activities were determined in the presence of 1−10 mM NAD and 140 μM propionaldehyde with an Aminco filter fluorometer after enzymes were incubated in 100 mM sodium phosphate (pH 7.4) at the desired temperature for 1 min and then assayed for remaining activity at 25 °C.

RESULTS

Expression. The expression and purification of the various mutant forms of the recombinantly expressed human mitochondrial ALDHs was performed essentially as was done for the native and Oriental variant of the enzyme *(5).* All proteins were purified to homogeneity as judged by SDS−PAGE (data not shown). Western blot analysis showed that the antibodies prepared against the native enzyme recognized mutant forms of the enzymes.

Kinetic Properties of the Arginine to Glutamine Mutants. Native ALDH, though functioning with half-of-the-site reactivity *(18, 19),* does not exhibit any form of cooperativity when either NAD or aldehyde was used as the variable substrate as indicated by the Hill coefficient of 1. It is not understood why the enzyme functions with just two active sites per tetramer, especially since there is a coenzyme binding site in each subunit *(6, 7).* Different and unexpected results were obtained when the R475Q mutant was investigated. For this mutant enzyme, the Lineweaver−Burk plots were nonlinear when NAD was the variable substrate. By fitting the data to the Hill equation, we obtained a K_M for NAD of 850 μM and a Hill coefficient of 1.8, as illustrated in Figure 1. This is compared to native enzyme that had a K_M of 37 μM and a Hill coefficient of 1. In contrast to the R475Q mutant, the behavior of the R264Q mutant was more nativelike, exhibiting a K_M for NAD of 60 μM and a Hill coefficient of 1. Thus, it appears that disrupting the non-conserved Arg475/Glu487 salt bond converted the enzyme from one that did not exhibit cooperativity in NAD binding to one that did. Disruption of the salt bond to the residue within the same subunit, Arg264/Glu487, did not grossly affect the properties of the mutant. Despite the changes in NAD interactions, the K_M for acetaldehyde did not change for either mutant and the values for k_{cat} decreased by less than 40% (Table 1).

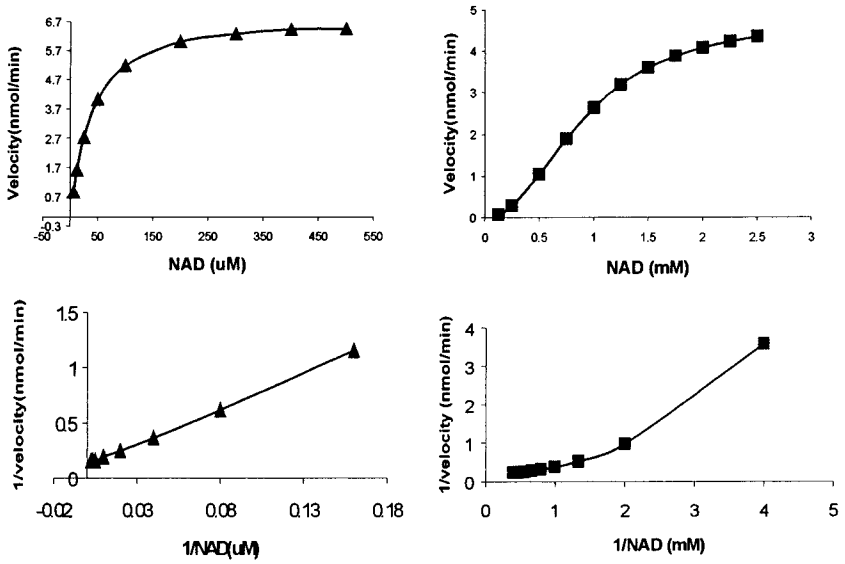

FIGURE 1: Saturation curves and Lineweaver−Burk double-reciprocal plots for the activity of the native (▲) and R475Q mutant (■) of human liver mitochondrial ALDH with NAD as the variable substrate. For native enzyme k_{cat} = 210 min^{-1}, K_m (NAD) = 37 μM, and Hill coefficient = 1.0, while for the R475Q mutant k_{cat} = 120 min^{-1}, K_m (NAD) = 850 μM, and Hill coefficient = 1.8.

Table 1: Kinetic Properties of the Native and the 475 and 264 Arginine Mutant Forms of Human Liver Mitochondrial ALDH

mutant	K_M NAD (μM)	k_{cat} (min^{-1})	Hill coefficient (n)
native	37	210	1.0
R475Q	850	120	1.8
R264Q	60	125	1.0
R475Q, R264Q	1300	nd	1.6
R475E	1300	18	2.0
R264E	740	12	1.0
R475E, R264E	16 000	4	nd[a]

[a] The activity was too low to determine accurately a Hill coefficient.

The rate-limiting step for human mitochondrial class 2 ALDH is deacylation *(20)*, and it appears that it also is for the Arg475 mutant. Chloroacetaldehyde was oxidized more rapidly than was acetaldehyde, and Mg^{2+} ions increased the specific activity of the enzyme. These are properties found when deacylation is the rate-limiting step (*21*). Furthermore, the pre-steady-state burst magnitude was the same as found with the native enzyme. Thus, despite the drastic changes in the binding of NAD, the overall catalytic mechanism of the enzyme was not altered (Table 2).

Since Glu487 interacts with the two arginine residues, a double mutant was prepared. This double mutant, R264Q/R475Q, had an even a greater K_M for NAD than did either single mutant and still showed cooperativity. The K_M value was nearly double that found for the R475Q mutant, reaching a value over 35 times that of the native enzyme (Table 1). It appeared that the final value was related to the product of the 20-fold increase with the 475 mutant and the 2-fold increase with the 264 mutant and not a simple additive effect of the two K_M values.

Kinetic Properties of Arginine to Glutamate Mutants. It was our goal to restore activity to the Oriental variant by restoring salt bonds between 487 and 264/475. Changing either arginine 264 or 475 to a glutamate proved to be deleterious for the activity of the enzyme. The new K_M values, tabulated in Table 1, show that these mutations increased the values by a factor of 20 and 50, respectively. Unlike the glutamine mutation where k_{cat} decreased by a factor of only 2, the values for the glutamate mutants were decreased by a factor of 10 compared to the native enzyme. The double mutant, R475E/R264E, again exhibited kinetic properties that were poorer than those found in the single mutant. The K_M for NAD increased over 600-fold. Thus, though the salt bond between Arg264 and Glu487 could be disrupted by having a glutamine present, negative charges at both residues 264 and 487 could not be well tolerated.

Kinetic Properties of Arginine Mutants of the Oriental Variant. To test for the importance of the salt bond and the possible repulsion that might have occurred in the Oriental variant, the residue at position 487 was also changed. We previously showed that the enzyme could well tolerate a glutamine at position 487 *(5)*. Its properties, as well as those of the Oriental variant, are presented in Table 3 along with those for the arginine 475 and 264 mutations made to the Oriental variant. No mutation of the arginine at 264 or 475 caused a restoration of the low activity exhibited by the Oriental variant. The K_M for NAD decreased from 7400 to 1500 μM when either or both of the arginine residues were converted to a glutamine while k_{cat} increased just 2-fold. In an attempt to restore the salt bond, the arginines were next converted to glutamates. Though there was some recovery

Table 2: Kinetic Constants of the Native and the R475Q Mutant Form of Human Liver Mitochondrial ALDH

	K_M (μM)		k_{cat} (min^{-1})		Hill coefficient (n)			
mutant	(NAD)	(Prop)[a]	0 mM Mg^{2+}	2 mM Mg^{2+}	0 mM Mg^{2+}	2 mM Mg^{2+}	ratio[b]	burst magnitude[c]
native	37	0.3	220	380	1.0	1.0	5.1	2.0
R475Q	850	0.6	120	210	1.8	2.0	4.8	2.1

[a] Prop is propionaldehyde. [b] Ratio is the ratio of dehydrogenase activity when chloroaldehyde and propionaldehyde were used as substrates. [c] The burst magnitude was expressed as the moles of NADH formation per mole of the tetrameric enzyme.

Table 3: Kinetic Properties of the 475 and 264 Arginine Mutants of the Oriental Variant of Human Liver Mitochondrial ALDH

mutant	K_M (NAD) (μM)	k_{cat} (min^{-1})	Hill coefficient (n)
E487K	7400	16	1
E487K, R475Q	1500	27	1.5
E487K, R264Q	1400	27	1
E487K, R264Q, R475Q	1500	nd	1.4
E487K, R475E	3200	40	1.5
E487K, R264E	700	31	1.0
E487K, R264E, R475E	7700	7	1.4
E487R	3700	6	1.0
E487R, R475E	2000	20	1.6
E487R, R264E	990	27	1.0
E487R, R475E, R264E	8100	2	nd[a]
E487Q	90	120	1.0

[a] The activity was too low to determine accurately a Hill coefficient.

Table 4: Esterase Activity of the Native and R475Q Mutant Forms of Human Liver Mitochondrial ALDH in the Absence and Presence of Coenzyme

mutant	k_{cat} (−coenzyme) (min^{-1})	$S_{0.5}$[a] (μM)	k_{cat} (+coenzyme) (min^{-1})	Hill coefficient (n)
		NAD		
Native	56	38	250	1.0
R475Q	2	1030	150	1.5
		NADH		
Native	56	3	180	1.0
R475Q	2	210	380	1.4
E487K	2	6	6	1.0

[a] $S_{0.5}$ is the concentration of coenzyme needed for half-maximal stimulation.

with the R264E mutant, the R475E proved to be even less active than the Oriental variant.

A last attempt to restore activity to the Oriental variant was made by reestablishing the salt bond between residues 487 and 475/264. Residue 487 in the Oriental variant was changed from a lysine to an arginine. The rationale was that if the glutamate in the active form of the enzyme could simultaneously make two salt bonds, then perhaps an arginine at 487 could make two salt bonds to glutamates placed at positions 264 and 475. As indicated by the data in Table 3, the E487R had properties very similar to those of the Oriental E487K variant. K_M for NAD increased dramatically and k_{cat} decreased to 5% that of the native enzyme. Some small recovery in K_M and k_{cat} of the Oriental variant was observed when either arginine at 264 or 475 were converted to a glutamate. However, the Hill coefficient for the 475 mutant remained greater than 1.5. The double arginine mutant of the Oriental variant had very little activity.

NAD and NADH Binding. Most of the mutants we previously investigated that had impaired binding of NAD did not exhibit impaired binding of NADH *(5, 17, 21)*. The binding of NADH to the R475Q mutant was investigated by first monitoring the fluorescence of enzyme-bound NADH. Unlike with the native or E487K mutant, no enhanced fluorescence could be observed when NADH and Arg475 mutants were mixed. This could have been a result of impaired binding or of a lack of enhanced fluorescence of bound NADH compared to free NADH. Since we could not measure directly the binding of NADH to the mutant enzyme, we choose to monitor its effect on the esterase reaction catalyzed by ALDH. In addition to the dehydrogenase reaction, ALDH catalyzes the hydrolysis of activated esters such as NPA *(22)*. While this reaction occurs in the absence of NAD(H), it has been shown that coenzyme will stimulate the rate of hydrolysis of the ester *(22)*. Though the rate of the dehydrogenase reaction was decreased by just 40%, there was essentially no esterase reactivity in the R475Q mutant. This implied that in the absence of coenzyme, Cys302 *(23)* was not functioning as a nucleophile, but in the presence of NAD, it could. From the data presented in Figure 2 and summarized in Table 4, it can be seen that NAD enhanced the rate of reaction. The concentration necessary for half-maximal activity was near that of the K_M found for NAD with the R475Q mutant enzyme. The Hill coefficient was 1.5, compared to 1 for the native enzyme, and the specific activity in the presence of a saturating concentration of NAD was half that of the native enzyme. This finding shows that the binary complex between enzyme and NAD

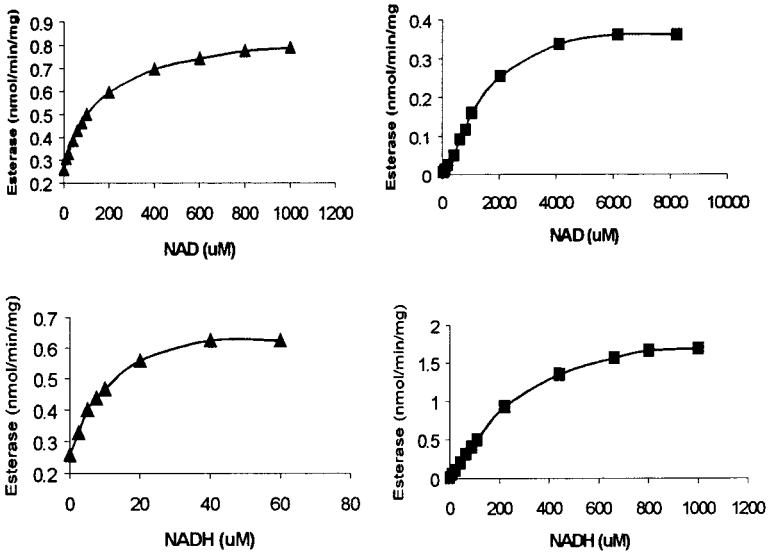

FIGURE 2: Esterase reaction of the native (▲) and the R475Q (■) mutant of human liver mitochondrial ALDH performed in the presence of coenzyme. Both NAD and NADH activated the esterase reaction showing that each coenzyme can bind to the apo-mutant enzyme in a cooperative manner.

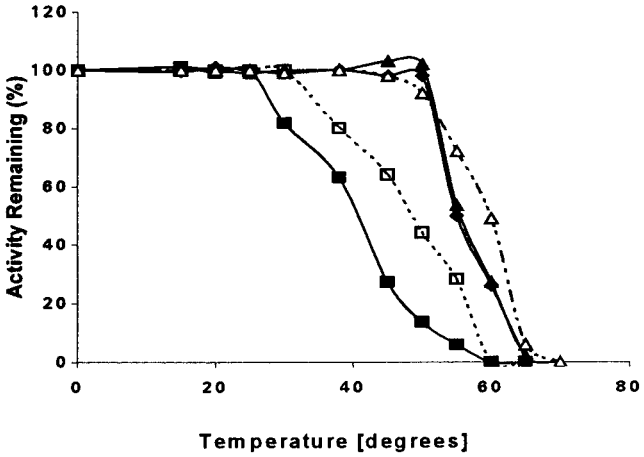

FIGURE 3: Thermal stability of native and mutant forms of human liver mitochondrial ALDH as determined by activity remaining as a function of temperature. Enzyme activity was determined after enzymes were incubated in the assay buffer at the desired temperature for 1 min. Native enzyme with NAD (△) and without NAD (▲), R475Q with NAD (□) and without NAD (■), and E487Q without NAD (◆).

functions normally and the mutant primarily impairs the binding of NAD and loses the ability to have the general base Glu268 *(20)* activate the nucleophilic residue Cys302.

NADH was found also to enhance the esterase reaction as shown in Figure 2. This property was used to determine if the R to Q mutation at position 475 altered NADH binding as it did NAD binding. The concentration of NADH needed for half-maximal stimulation increased from 5 μM in the native enzyme to over 200 μM in the mutant with a Hill coefficient of 1.4. The point mutation at position 475 affected the binding of both NAD and NADH to aldehyde dehydrogenase. Thus, it appeared that there could be some structural alteration of the mutant enzyme compared to the native enzyme that is restored upon coenzyme binding.

Thermostability of Mutants of ALDH. To test for possible structural alterations between the 475 mutants and the native enzyme, the stability of the enzymes as a function of temperature was measured. First it was found that the R475Q mutant would lose all its activity when incubated at 50 °C for 2 min. In contrast, the native enzyme lost only 10% of its activity under the same conditions. Next, activity remaining after a 1 min incubation at different temperatures was determined. The native enzyme lost 50% activity at 55 °C, while the R475Q mutant lost half the activity at 40 °C (Figure 3). The greater loss of activity at elevated temperatures of the R475Q mutant could be related to the disruption of the salt bond between Glu 487 and Arg475 or to the mere presence of the glutamine residue at position 475. The stability of the Oriental variant (data not shown) and the E487Q mutant was measured and found to be similar to that of the native enzyme. Thus, the decreased stability of the R475Q mutant was not simply due to the disruption of the salt bond but was due to the loss of the arginine itself. NAD was found to cause the R475Q mutant to become more stable, while it hardly affected the thermal stability of the native enzyme. Though the temperature where half the enzyme denatured was still lower than that found for the native binary complex, it can be concluded that the binary complex of R475Q is more nativelike than is the apoenzyme.

DISCUSSION

The only well-studied variant of human aldehyde dehydrogenase is the one found in many Asian people. The Oriental variant of the class 2 mitochondrial homotetrameric enzyme has a lysine at position 487 rather than the glutamate found in non-Asian people. Investigators employing gel assays reported that they could not detect any catalytic activity associated with the Oriental variant *(4)*. In subsequent studies we found that the recombinantly expressed E487K form possessed about 10% the specific activity of the native enzyme (5). More striking, perhaps, was finding that the K_M and K_{ia} for NAD increased dramatically, while the K_d for NADH was hardly affected. The glutamate residue at position 487 is not conserved in all active forms of the enzyme. No known form of the enzyme has a lysine or arginine at this position, but some have a glutamine or a histidine at this position *(24, 25)*. We showed that converting the glutamate to a glutamine did not drastically alter the properties of the class 2 human mitochondrial enzyme *(5)*. Thus, it is not the loss of the glutamate but the presence of the lysine that caused the enzyme to have such drastically altered properties.

Others found that people who phenotyped as possessing the Oriental variant actually were heterozygotic in that they had genes coding for both the active E-form and the inactive K-form of the enzyme *(26)*. We showed that these people actually possessed both forms of the enzyme and then, by using a coexpression system, showed that the heterotetrameric form composed of active E- and inactive K-subunits had properties more similar to those of the K-enzyme *(8)*. Thus, the low-activity K-subunit also affected the properties of the E-subunit in a heterotetramer. This was the first evidence that communication between subunits could occur, though it was known that the enzyme did function with half-of-the-site reactivity *(18)*.

Prior to knowing the structure of the enzyme, it was not possible to predict why the K-subunit showed dominance over the E-subunit. We rationalized, though, that the alteration of binding of NAD but not of NADH could be related to the proximity of the residue at position 487 to the positively charged nicotinamide ring of NAD. After the structure of enzyme was determined, a possible mechanism could be proposed as to why the lysine substitution at position 487 caused the properties of the enzyme to change. Arginines at positions 475 and 264 formed a salt bond with the glutamate that was normally at position 487 *(6)*. One arginine, 264, is located in the same subunit as is the residue at position 487, while the other arginine was in the subunit that formed the dimer pair. We proposed that it was the disruption of this salt bond that existed between subunits that allowed the K-subunit to affect the other subunit. These points are illustrated in Figure 4. We have not yet obtained usable crystals of the Oriental variant to test these hypotheses, so we embarked upon a mutational analysis to test for its validity.

It was an unexpected finding that a change made to the arginine at position 475 would convert a noncooperative enzyme to one that exhibited positive cooperativity. The purpose of making the arginine mutants was to test the hypothesis that disruption of the salt bond between either Arg475 and/or Arg264 and Glu487 was the reason that the Oriental variant had properties different from those of the

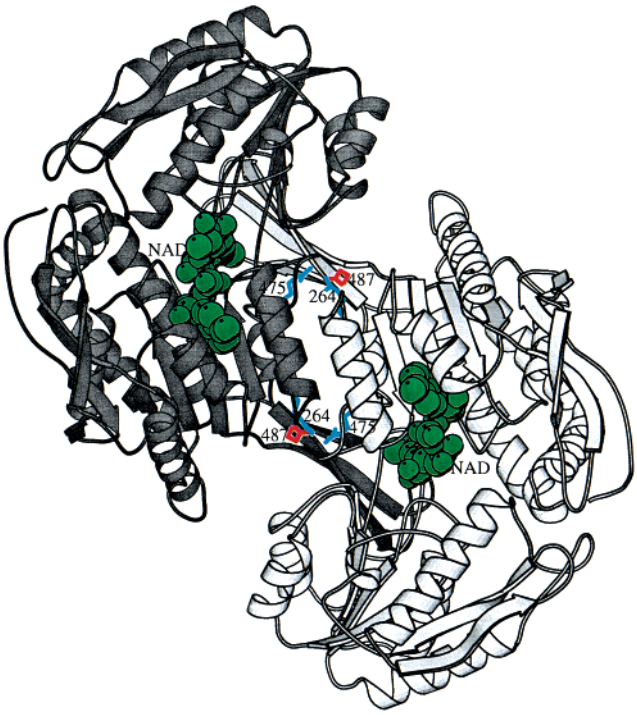

FIGURE 4: Ribbon diagram of a dimer of human liver mitochondrial ALDH subunits. Each subunit is colored a different shade of gray. The side chains of Arg264, Arg475 (cyan), and Glu487 (red) are labeled. The bound molecules of NAD are represented in space-filling spheres (green).

glutamate-containing enzyme. A mutation to Arg264 proved to have little effect on the enzyme, in contrast to mutation of Arg475. However, an attempt to restore the salt bond by making a R475E mutation in the context of the Oriental variant proved to be unsuccessful. This mutant had essentially the same properties as did the R475Q mutant. Since it was not possible to restore nativelike properties by having a salt-bonding pair between residues 475 and 487, it can be concluded that the removal of the arginine residue at position 475 caused the alteration in properties of the Arg475 mutants.

The finding that R475Q had a Hill coefficient of nearly 2 shows that there was positive cooperativity associated with NAD binding, though the mutant still functioned with half-of-the-site reactivity. Because the enzyme catalyzes an essentially irreversible reaction, it was not possible to determine if the mutation would cause a change in NADH binding in a dehydrogenase reaction. It was possible to use the effect of coenzyme on the esterase reaction to show that NADH binding was also affected. Thus, a mutation to Arg475, unlike other mutations we made to the enzyme, caused a change in both NAD and NADH binding to occur. No other mutant thus far studied showed any evidence for cooperativity, implying that Arg475 alone is responsible for the altered properties. The fact that R475Q was less stable than the native enzyme, but that stability could be restored in the presence of NAD, may be interpreted as implying that the conformation of the apo form of the R475Q mutant is very different from that of the apo form of the native enzyme. When, however, the mutant enzyme is saturated with NAD, the properties of the mutant and native enzyme become similar. The binding of NAD apparently converts R475Q into a nativelike enzyme. The stability study showed that it was not the simple breaking of the salt bond between residues

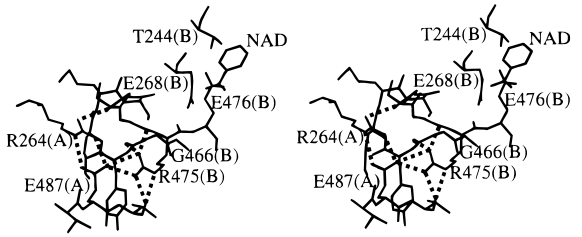

FIGURE 5: Stereo diagram showing the structure of human liver mitochondrial ALDH in the vicinity of Arg475. Hydrogen-bonding interactions are indicated as dashed lines. An additional hydrogen-bonding interaction occurs between the side chain of Arg264 and Gly467 (2.9 Å) but is not shown for the sake of clarity of presentation.

487 and 475 that led to the thermal instability of the mutant. E487Q, a mutant that could only form a hydrogen bond with Arg475, has similar stability as the native enzyme. The evidence reported here shows that the changes in kinetic behavior and in stability occur not by simply breaking the salt bond with Arg475 but perhaps are associated with changes in the position of residue 475, which then affect other portions of the enzyme.

Additional evidence to suggest that the R475Q and native enzyme became more similar when saturated with coenzyme was the specific activity of the enzymes. While the mutation to Arg475 caused a drastic change in coenzyme binding behavior, there was only a 40% reduction in the specific activity of the dehydrogenase reaction and the esterase velocity reached nativelike levels in the presence of NAD. The fact that there was no measurable esterase reaction in the absence of added coenzyme for the R475Q mutant leads us to propose that the movement of R475 affects the reactivity of the active site nucleophile, Cys302. This could occur if the conformation of the active-site region was altered in the apoenzyme and was restored in the binary complex.

Arg475 makes contact with different residues in the structure of the enzyme. In addition to the interactions with the side chain of Glu487 in the neighboring subunit, Arg475 also forms hydrogen-bonding interactions with the main-chain peptide carbonyl oxygen atoms of residues 474 and 467 in its own subunit and with the peptide carbonyl oxygen atom of residue 488 in the neighboring subunit (Figure 5). Thus, its positioning and the nature of its interactions appear to stabilize a large area of the structure, including the loop comprising residues 463−478. This loop forms the "floor" of the active site, and changes in its conformation could undoubtedly influence catalytic activity and possibly also the reactivity of Cys302. The fact that compensating charges to Lys487 in the Oriental variant could not restore nativelike properties is consistent with the nature of the interactions contributed by Arg475. The guanidino group of Arg475 donates a total of six hydrogen-bonding interactions, something neither a glutamate nor a glutamine residue could perform. In light of these results and the local structure surrounding residue 475, it is not surprising that mutations at 475 might also decrease the thermal stability of the enzyme. Much of the conformational stability of this region is created through the hydrogen-bonding interactions between the arginine side chain and main-chain atoms of the surrounding polypeptide.

What is not yet clear is why the enzyme obeys cooperative binding kinetics and the thermal stability is restored to

nativelike properties in the presence of NAD. One possibility is that Arg264 may sense the presence of cofactor since it is located near the end of the helix comprising residues 246−262. This helix forms both part of the adenine binding cleft and part of the dimeric interface by interacting with the same helix in the adjacent subunit. Binding of cofactor in one subunit may induce some conformational transition that is then translated to the other subunit, promoting easier binding of the next cofactor and apparently also stabilizing the whole tetrameric structure. We originally suggested that this helix may act as the sensor for half-of-the-site reactivity *(6)*, and its role in this mutant form of ALDH may also be expanded to include cooperative interactions. However, mutating this arginine residue did not convert the enzyme into one that exhibits cooperativity in NAD binding as it did when Arg475 was mutated. This could be explained by the fact that Arg264 appears to play only a minor role in stabilizing the loop comprising residues 466−476. The guanidino group of Arg264 forms two hydrogen bonds with the peptide carbonyl oxygens of residues 472 and 467 in the opposite subunit (Figure 5). These two interactions appear to be less critical to the conformational stability of the enzyme as judged by the structure and by the results of mutations to position 264. However, when the interactions contributed by Arg475 are disrupted, the interactions contributed by Arg264 may become important but are only sufficient to stabilize the structure once the active site has been partially stabilized by the binding of NAD. Cooperativity could then arise because the binding of NAD to one subunit would stabilize both the position of Arg264 and the helix that comprises the NAD binding site in the other subunit.

In this study we found that a mutation to just one residue that was involved in intersubunit interactions was sufficient to produce an enzyme that exhibits cooperativity in substrate binding. There are other examples of this phenomenon in the literature. These include studies with glutathione reductase *(27)*, phosphofructokinase *(28)*, and pyruvate kinase *(29)*. For each, the Hill coefficient increased just as we have shown here for NAD binding to ALDH. There are two generally accepted models to explain cooperativity. One is that a conformational change occurs when ligand binds; the other is that there are two conformational states of the enzyme, R and T, and the binding of ligand stabilizes one conformation *(30)*. The mechanism for the mutant-induced cooperativity for ALDH is not yet understood. It is known that there is disorder in the coenzyme structure in the tetrameric ALDH in that the nicotinamide ring cannot be located in every subunit while the adenosine portion of NAD can be found in each subunit *(7, 31)*. Finding that the R475Q mutant became more thermally stable when NAD was present leads us to suggest that the presence of NAD could induce a structural change in the subunits. Only when saturated with coenzyme would the mutant have a nativelike structure that would lead to nativelike kinetic and thermal properties.

Though there is an interaction between residues 487 and 475, mutations to these residues produced enzymes with very different properties. No mutation to 487 produced an enzyme that exhibited positive cooperativity in substrate binding. Furthermore, the Oriental variant had only 10% the specific activity of the Glu487 form, while R475Q had 60% of the activity. It was not possible to restore nativelike properties to the E487K Oriental variant by restoring salt bonds between this residue and those located at 264 and 475. Mutations such as changing the arginines to glutamates still produced a low-activity enzyme. Even the E487R mutant, which was similar to the Oriental variant, could not be made to have native properties when 264 and 475 were converted to acidic residues. Thus, it was not just the loss of the salt bond that led to the loss of activity of the Oriental variant. It appears likely that the presence of a positive charge at position 487 forces Arg475 to move. This movement alone cannot be the only reason that the Oriental variant behaves as it does.

Though this study was originally designed to determine why the Oriental variant of ALDH had altered properties, we discovered that the nonconserved arginine located at position 475 was responsible for preserving subunit interactions in the tetrameric enzyme. Few detailed kinetic studies of forms of ALDH that do not have an arginine at the equivalent of position 475 have been reported other than for the dimeric class 3 enzyme (*24*). It is not known if tetrameric forms of the enzyme lacking an arginine at the equivalent of position 475 would show traditional subunit interactions or have positive Hill coefficients for the binding of coenzyme.

ACKNOWLEDGMENT

We thank Rachel Abeles, a summer intern, for assisting with the initial studies on the arginine 475 mutant.

REFERENCES

1. Svanas, G. W., and Weiner, H. (1985) *Alcohol 2*, 111−115.
2. Yoshida, A., Huang, I.-Y., and Ikawa, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 258−261.
3. Hempel, J., Kaiser, R., and Jörnvall, H. (1984) *FEBS Lett. 173*, 367−373.
4. Ikawa, M., Impraim, C. C., Wang, G., and Yoshida, A. (1983) *J. Biol. Chem. 258*, 6282−6287.
5. Farrés, J., Wang, X., Takahashi, K., Cunningham, S. J., Wang, T. T., and Weiner, H. (1994) *J. Biol. Chem. 269*, 13854−13860.
6. Steinmetz, C. G., Xie, P. G., Weiner, H., and Hurley, T. D. (1997) *Structure* 5, 701−711.
7. Ni, L., Zhou, J., Hurley, T. D., and Weiner, H. (1999) *Protein Sci. 8*, 2784−2790.
8. Wang, X., Sheikh, S., Saigal, D., Robinson, L., and Weiner, H. (1996) *J. Biol. Chem. 271*, 31172−31178.
9. Tabor, S., and Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074−1078.
10. Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113−130.
11. Zheng, C. F., and Weiner, H. (1993) *Adv. Exp. Med. Biol. 328*, 73−79.
12. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 51−59.
13. Ghenbot, G., and Weiner, H. (1992) *Protein Expressioin Purif. 3*, 470−478.
14. Jeng, J. J., and Weiner, H. (1991) *Arch. Biochem. Biophys. 289*, 214−222.
15. Laemmli, U. K. (1970) *Nature 227*, 680−685.
16. Takahashi, K., and Weiner, H. (1981) *Biochemistry 20*, 2720−2726.
17. Rout, U. K., and Weiner, H. (1994) *Biochemistry 33*, 8955−8961.
18. Sanny, C. G., and Weiner, H. (1987) *Biochem. J. 242*, 499−503.
19. Weiner, H., Hu, J. H., and Sanny, C. G. (1976) *J. Biol. Chem. 251*, 3853−3855.

20. Wang, X., and Weiner, H. (1995) *Biochemistry 34*, 237−243.
21. Ni, L., Sheikh, S., and Weiner, H. (1997) *J. Biol. Chem. 272*, 18823−18826.
22. Feldman, R. I., and Weiner, H. (1972) *J. Biol. Chem. 247*, 267−272.
23. Farrés, J., Wang, T. Y., Cunningham, S. J., and Weiner, H. (1995) *Biochemistry 34*, 2592−2598.
24. Mann, C. J., and Weiner, H. (1999) *Protein Sci. 8*, 1922−9.
25. Perozich, J., Nicholas, H., Wang, B. C., Lindahl, R., and Hempel, J. (1999) *Protein Sci. 8*, 37−46.
26. Crabb, D. W., Edenberg, H. J., Bosron, W. F., and Li, T.-K. (1989) *J. Clin. Invest. 83*, 314−316.
27. Scrutton, N. S., Deonarain, M. P., Berry, A., and Perham, R. N. (1992) *Science 258*, 1140−3.
28. Auzat, I., Le Bras, G., and Garel, J. R. (1995) *J. Mol. Biol. 246*, 248−53.
29. Ikeda, Y., Tanaka, T., and Noguchi, T. (1997) *J. Biol. Chem. 272*, 20495−501.
30. Stryer, L. (1995) in *Biochemistry*, 4th ed., pp 166−168 and 198−199, W. H. Freeman and Co., New York.
31. Moore, S. A., Baker, H. M., Blythe, T. J., Kitson, K. E., Kitson, T. M., and Baker, E. N. (1998) *Structure 6*, 1541−1551.

BI000028N