The Potential Roles of the Conserved Amino Acids in Human Liver Mitochondrial Aldehyde Dehydrogenase*

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The sequence alignment of all known aldehyde dehydrogenases showed that only 23 residues were completely conserved (Hempel, J., Nicholas, H., and Lindahl, R. (1993) Protein Sci. 2, 1890–1900). Of these 14 were glycines and prolines. Site-directed mutagenesis showed that Cys302 was the essential nucleophile and that Glu268 was the general base necessary to activate Cys302 for both the dehydrogenase and esterase reaction. Here we report the mutational analysis of other conserved residues possessing reactive side chains Arg84, Lys192, Thr384, Glu399, and Ser471, along with partially conserved Glu398 and Lys489, to determine their involvement in the catalytic process and correlate these findings with the known structure of mitochondrial ALDH (Steinmetz, C. G., Xie, P.-G., Weiner, H., and Hurley, T. D. (1997) Structure 5, 701–711). No residue was found to be absolutely essential, but all the mutations caused a decrease in the specific activity of the enzyme. None of the mutations affected the Km for aldehyde significantly, although the rate constant calculated for aldehyde binding was decreased. The Km and dissociation constant (Kd) for NAD+ increased significantly for K192Q and S471A compared with the native enzyme. Mutations of only Lys192 and Glu399, both NAD+-ribose binding residues, led to a change in the rate-limiting step such that hydride transfer became rate-limiting, not deacylation. Esterase activity of all mutants decreased even though mutations affected different catalytic steps in the dehydrogenase reaction.

Oxidation of toxic aldehydes to their corresponding acids is primarily catalyzed by aldehyde dehydrogenase (ALDH).1 During the last two decades, several ALDHs from different organisms were discovered. By aligning the sequences of 16 known ALDHs, it was found that only 23 amino acids were completely conserved (1). More recently, Vasiлиu et al. (2) classified 26 mammalian ALDHs based on divergent evolution and reported that same residues were conserved. We undertook a mutagenesis approach to investigate the role of the conserved residues in the human mitochondrial ALDH, which possessed a functional side chain. Since completion of the study, the three-dimensional structure of the corresponding beef liver enzyme has been determined to 2.65 Å (3).

Prior to the advent of molecular biological techniques, chemical modifications were used to identify the components of the active site of the enzyme. Classical sulfhydryl reagents inactivated the enzyme (4–6). Iodoacetamide was shown to modify Cys302 in human ALDH (4) while using protection studies; our laboratory reported that Cys489 was a component of the active site of the horse liver enzyme (7). Two other residues were identified by chemical modifications as being possibly involved in the catalytic process. These were Glu268 and Ser74 in the human (8) and sheep liver (9) enzymes, respectively. It was shown later by site-directed mutagenesis studies that Cys302 is a nucleophile (10) and Glu268 (11) functions as a general base during catalysis. However, Ser74 (12) and Cys489/Cys162 (10) were found to be not essential for the catalytic reaction.

The kinetics of ALDH was found to follow an ordered sequential mechanism where NAD+ binds first followed by aldehyde (13). The reaction involves both acylation and deacylation steps during the oxidation of aldehyde to acid, or hydrolysis of nitrophenyl acetate. It was proposed that deacylation (k3) (Fig. 1) was rate-limiting for horse liver ALDH2 (13, 14) for the dehydrogenase reaction, while acylation (k1) was rate-limiting for the esterase reaction. Here we report the properties of human ALDH2 mutant enzymes produced by replacing the conserved amino acids possessing a reactive side chain with different residues. The purpose of this study is to understand the potential role of the conserved residues in the catalytic mechanism of ALDH and to relate the effects to the now known structure. In the accompanying paper, a detailed analysis of the Lys192 and Glu399 mutants that caused a change in the rate-limiting step of the enzyme will be presented (15).

Experimental Procedures

Materials—NAD+ and NADH were purchased from Sigma; Sequenase version 2.0 kit was obtained from United States Biochemical Corp.; propionaldehyde, chloroacetaldehyde, and p-nitrophenyl acetate were from Aldrich; Magic MiniPreps DNA purification system and T4 DNA ligase were from Promega Corp.; alkaline phosphatase-conjugated goat anti-rabbit IgG and Muta-Gene DNA ligase were from Promega Corp.; alkaline phosphatase-conjugated goat anti-rabbit IgG and Muta-Gene in vitro mutagenesis kit were from Bio-Rad; GeneClean kit was from Bio 101, Inc.; [α−32P]dATP was from Amersham Corp.; and the restriction enzymes used were from either New England Biolabs or Promega Corp.

Cells and Plasmids—Native and mutant ALDH cDNAs were cloned on pT7-7 expression vector, a derivative of pT7-1 (16), and expressed in Escherichia coli strain BL21 (DE3) pLysS (17), as reported previously (18).

Oligonucleotide-directed Mutagenesis—To construct the human ALDH2 R84E/Q, K192Q, T384A/S, K489E/Q, S471A/T, E398K, and E399Q mutants, the oligonucleotide primers containing the mutation were used for site-directed mutagenesis with the Mutagene Kit following the manufacturer’s instructions. The mutant colonies were selected by sequencing using the dideoxynucleotide chain-termination method.

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**RESULTS**

**Expression and Purification of Human Native and Mutant Forms of ALDH2**—Recombinantly expressed native and the mutant forms of human liver ALDH2 were purified to homogeneity by established methods, as judged by SDS-PAGE followed by Coomassie Blue staining (11, 18, 21). The expression of the native and mutant enzymes was also verified by Western blotting with antibodies against the beef liver ALDH2. R84Q, K192Q, T384A/S, K489Q, S471A/T, E398K, and E399Q mutants were expressed at a level similar to the native ALDH2, but, R84E, K192E, and K489E mutants were present at a much lower level. The expression of the S471T mutant was similar to that of the native enzyme, but this mutant was less stable. After purification the dehydrogenase activity of S471T decreased to 50% if kept overnight while the others were stable for several days at 4 °C in the presence of 0.1 mM dithiothreitol.

**Kinetic Properties of the Human ALDH2 Mutants**—All the mutants examined were found to have lower activity compared with the native enzyme. Replacement of conserved residues by neutral amino acids had less effect on the activity than when oppositely charged amino acids were used (Table I). No major changes in the $K_m$ for propionaldehyde was found with the mutant enzymes, when compared with the native enzyme, as was observed for other mutants we previously characterized (11, 12, 21). The exception was with C302S, where the $K_m$ for propionaldehyde increased several thousandfold (10). A significant variation, however, was observed when the $K_m$ for NAD$^+$ was determined. In T384A, E398K, E399Q, and K489Q, the $K_m$ values obtained were within a factor of 9–8-fold higher than native enzyme. The values increased to approximately 100 and 50 times the native value for K192Q and S471A/T mutants, respectively. Other mutants, R84E/Q, T384A/S, and K489Q, and K489Q had $K_m$ for NAD$^+$ similar to that found with the native enzyme. However, by comparing the second order rate constant values ($V_{max}/K_m$), it was observed that the rate constant was significantly lower with all the mutants except for R84Q, compared with the native enzyme.

**NAD$^+$ and NADH Binding to ALDH2 Mutants Possessing Increased $K_m$ for NAD$^+$**—Earlier kinetic investigations suggested that the ALDH-catalyzed reaction followed a sequential burst of NADH formation at 400 nm in the presence of 0.1 mM dithiothreitol. A significant variation, however, was observed when the $K_m$ for NAD$^+$ was determined. In T384A, E398K, E399Q, and K489Q, the $K_m$ values obtained were within a factor of 9–8-fold higher than native enzyme. The values increased to approximately 100 and 50 times the native value for K192Q and S471A/T mutants, respectively. Other mutants, R84E/Q, T384A/S, and K489Q, and K489Q had $K_m$ for NAD$^+$ similar to that found with the native enzyme. However, by comparing the second order rate constant values ($V_{max}/K_m$), it was observed that the rate constant was significantly lower with all the mutants except for R84Q, compared with the native enzyme.

**Determination of Protein Concentration**—The protein concentration was determined with the Bio-Rad protein assay kit, using bovine serum albumin as a standard.

**TABLE I**

| Enzyme | $K_m$ (NAD$^+$) | $K_m$ (Prop) | $k_{cat}$/|$K_m$ (NAD$^+$) | $k_{cat}$/|$K_m$ (Prop) | $k_{cat}$/|$K_m$ (Chloro) | $k_{cat}$/|$K_m$ (Chloro)/prop | Burst | Rate limiting |
|--------|----------------|-------------|-----------|----------------|-------------|-------------|-------------|-----------|---------------------|------|-------------|
| Native | 28             | 0.53        | 180       | 6.4            | 340         | 700         | 3.9         | Yes       | k$_7$            |      | ND          |
| R84E   | 27             | 0.30        | 3.1       | 11             | 10          | 15          | 4.8         | ND        | k$_7$            |      | ND          |
| R84Q   | 32             | 1.3         | 57        | 1.8            | 44          | 210         | 3.7         | Yes       | k$_7$            |      | ND          |
| K192Q  | 360            | 3.5         | 35        | 0.01           | 10          | 17          | 0.5         | No        | k$_7$            |      | ND          |
| T384A  | 160            | 0.92        | 11        | 0.07           | 12          | 28          | 2.5         | Yes       | k$_7$            |      | ND          |
| T384S  | 50             | 0.64        | 27        | 0.54           | 42          | 84          | 3.1         | Yes       | k$_7$            |      | ND          |
| E398K  | 140            | 0.34        | 65        | 0.47           | 190         | 210         | 3.3         | Yes       | k$_7$            |      | ND          |
| E399Q  | 120            | 0.27        | 21        | 0.18           | 78          | 14          | 0.7         | No        | k$_5$            |      | ND          |
| S471A  | 1470           | 0.22        | 28        | 0.02           | 1370        | 99          | 3.5         | Yes       | k$_7$            |      | ND          |
| S471T  | 1680           | 0.47        | 4.6       | 0.003          | 9.8         | 15          | 3.3         | Yes       | k$_7$            |      | ND          |
| K489E  | 45             | 0.60        | 5.5       | 0.12           | 9.2         | 17          | 3.1         | ND        | k$_7$            |      | ND          |
| K489Q  | 220            | 0.50        | 70        | 0.31           | 140         | 200         | 2.9         | Yes       | k$_7$            |      | ND          |

*All residues except Glu$^{398}$ and Lys$^{489}$ were completely conserved among all known ALDHs.

*Though individual average values were calculated, the $K_m$ for aldehyde mostly ranged from 0.22 to 0.92 with an average value of 0.48. This value was used to produce the data presented in Fig. 3.

*$k_{cat}$/|$K_m$ (Prop) and $k_{cat}$/|$K_m$ (Chloro) refers to dehydrogenase activity of the enzyme with propionaldehyde and chloroaacetaldheyde as the substrates, respectively.

Burst magnitude range from 1.5 to 2.0 mol of NADH/mol of enzyme. The lower limit of detection was 0.1.

*Rate limiting.

**Pre-steady State Burst of NADH Formation**—The pre-steady state burst magnitude of NADH formation was determined with a Hitachi 2000 Fluorescence Spectrophotometer (12, 21). Enzyme and NAD$^+$ were incubated in 100 mM sodium phosphate (pH 7.4) to establish a fluorescence baseline. Concentrations of NAD$^+$ were 1–7 mM for the native and different mutant enzymes. At a time called zero, propionaldehyde (140 μM) was added to initiate the reaction. The extrapolated line intersecting at time zero gave the magnitude of the burst of NADH formation. By calibrating the fluorometer with various concentrations of NADH, it was possible to calculate the moles of NADH produced prior to the steady state rate of NADH formation (12, 18).

**Determination of the Dissociation Constant ($K_d$) for NADH**—The dissociation constant of NADH was determined by measuring the rate of increase in fluorescence at 400 nm in the presence of 14 μM NADH, it was possible to calculate the moles of NADH produced prior to the steady state rate of NADH formation. By calibrating the fluorometer with various concentrations of NADH, it was possible to calculate the moles of NADH produced prior to the steady state rate of NADH formation (12, 18).
TABLE II

Determination of NAD$^+$ and NADH binding constants for the native, K192Q, and S471A mutant forms of human liver mitochondrial aldehyde dehydrogenase (ALDH2)

| Kinetic constant | Native | K192Q | S471A |
|------------------|--------|-------|-------|
| $k_{cat}$ (min$^{-1}$) | 200    | 44    | 22    |
| $K_a$ (µM)       | 53     | 2300  | 1480  |
| $K_d$ (µM)       | 0.5    | 3.4   | 0.15  |
| $k_1$ (µM min$^{-1}$) | 3.9    | 0.019 | 0.015 |
| $k_2$ (µM min$^{-1}$) | 31     | 11    | 4.2   |
| $K_h$ (µM)       | 340    | 10    | 130   |
| $K_q$ (µM)       | 11     | 590   | 280   |
| $K_f$ (µM)       | 3      | 9     | 17    |

Mechanism with NAD$^+$ being the first substrate followed by aldehyde binding (13). The dissociation constant ($K_a$) for NAD$^+$ with ALDH2 was determined by bisubstrate kinetic analysis (11, 12, 21). Several concentrations of NAD$^+$ and propionaldehyde were used for the analysis of the data. The kinetic constants were calculated by the Dalziel graphical method (23). The value of $K_a$ increased to more than 50- and 25-fold in K192Q and S471A, respectively, compared with the native enzyme (Table II). The value $k_{cat}/K_{cat(NAD^+)}$ is $k_1$ (on velocity), which decreased 200-fold compared with the native enzyme. Similarly, $k_2$, the off velocity for NAD$^+$ dissociation, was also affected.

The dissociation constant ($K_q$) for NADH was calculated by measuring the increase in fluorescence observed when NADH binds to the enzyme (22). Analysis of the NADH binding data showed a very small increase in the dissociation constant with the mutant enzymes and no difference in the binding stoichiometry. These results are similar to the previously characterized S74A and E487K mutants, where the $K_a$ and $K_h$ for NAD$^+$ were increased significantly but the binding for NADH was not affected (12, 21).

Determination of the Rate-limiting Step for the Mutant Enzymes—The mechanism of ALDH involves several intermediates, as shown in Fig. 1. It was observed that a pre-steady state burst of 2 mol of NADH/mol of tetrameric enzyme occurred with the recombinantly expressed human mitochondrial ALDH2 (11). In addition, the $V_{max}$ value was found to be dependent on the nature of the substrate (10, 24), suggesting deacylation ($k_2$) was the rate-limiting step for the native enzyme. For most mutants a burst magnitude of essentially 2 mol of NADH/mol of enzyme was found, except with K192Q and E399Q, where no pre-steady state burst of NADH formation and a decreased rate of the reaction with chloroacetaldehyde was found, hydrate transfer appears to be the rate-limiting step ($k_2$).

**Determination of Esterase Activity of the Mutant Enzymes**—In addition to dehydrogenase activity, aldehyde dehydrogenase also possesses esterase activity (13, 25, 26). The reaction scheme for the esterase reaction is shown in Fig. 1. The mechanism for both the esterase and dehydrogenase reaction remains essentially the same except that the hydride transfer step is not involved in the esterase reaction. Although the esterase activity was decreased for the mutants, a relatively higher esterase activity was found, compared with their respective dehydrogenase activity (Table III). In all mutants the esterase activity was stimulated 2–7-fold by NAD$^+$.

**D I S C U S S I O N**

When we initiated the project to elucidate the role of the conserved amino acids found in all aldehyde dehydrogenases, no structure of the enzyme was known. After we completed the mutational analysis, the structure of the dimeric class three enzyme (27) and the beef liver mitochondria enzyme (3) were determined in the presence and absence of NAD$^+$. From the latter structure, it is apparent that only residues Lys$^{192}$ and Glu$^{399}$ are in contact with the coenzyme, and that none of the other conserved residues make contact with the coenzyme or other components of the active site. Fig. 2 illustrates the location of each conserved residue in the subunit as well as a few others we found to affect activity. The enzyme is actually a pair of dimers, as shown elsewhere (3).

All the residues investigated were conserved or at least conserved in all mammalian ALDHs. Mutation of Ser$^{71}$ to a threonine reduced the $k_{cat}$ to less than 5% of the value of the native enzyme. Converting the residue to an alanine produced an enzyme which retained 15% of the specific activity. Most surprisingly was to find that mutation of the two residues that interacted directly with the coenzyme did not more drastically affect the specific activity of the enzyme. In fact, the $K_a$ for NAD$^+$ was increased only when Lys$^{192}$ was converted to a glutamine (100-fold). Mutation of the other ribose binding ligand, Glu$^{399}$, caused the $K_m$ for NAD$^+$ to increase just 4-fold. In contrast, Ser$^{71}$, which is located approximately 9 Å from the coenzyme, caused the $K_a$ for NAD$^+$ to increase over 50-fold.

The effect is related to binding, for the $K_a$ values for NAD$^+$ to K192Q and S471A increased over 50- and 25-fold, respectively, as shown in Table II. While the binding of NAD$^+$ was affected by mutations to Lys$^{192}$ and Ser$^{71}$, the interaction of NADH was not. Its value increased only 3- and 6-fold, respectively.

Although none of the charged residues investigated in this study can be deemed essential, changing a residue to one with
TABLE III
Mutations of Conserved Residues in ALDH

| Enzyme | $k_{cat}$ (- NAD$^+$) | $k_{cat}$ (+ NAD$^+$) | Ratio |
|--------|----------------------|----------------------|------|
| Native | 37                   | 190                  | 5.0  |
| R84E   | 3.3                  | 4.4                  | 1.3  |
| R84Q   | 17                   | 2.4                  | 5.3  |
| K192Q  | 15                   | 61                   | 4.0  |
| T384A  | 5.5                  | 37                   | 6.8  |
| T384S  | 11                   | 24                   | 2.2  |
| E398K  | 19                   | 100                  | 5.3  |
| E399Q  | 25                   | 50                   | 2.0  |
| S471A  | 8.6                  | 33                   | 4.0  |
| K489Q  | 21                   | 150                  | 7.3  |

$^a$ The unit of the esterase activity is expressed in terms of min$^{-1}$. $^b$ Esterase activity was determined in the presence of 1 mM NAD$^+$ for native, R84E/Q, T384A/S, and K489Q mutants; 3 mM NAD$^+$ for E398K and E399Q mutants; 5 mM and 7 mM NAD$^+$ for S471A and K192Q mutants, respectively.

$^c$ Ratio of esterase activity in the presence and absence of NAD$^+$.

FIG. 2. The structure of one subunit of bovine liver mitochondrial ALDH. The various conserved and partially conserved residues with a reactive side chain are shown along with NAD$^+$. Lys$^{489}$ caps an a helix in another subunit, indicated by a. Glu$^{487}$ binds to an arginine in that subunit, indicated by b.

the opposite charge greatly affected the enzyme activity. This can be illustrated by the fact that K489E had only 3% of the specific activity of the native enzyme while K489Q had nearly 40%. Similarly, R84E had just 2% activity while the Gln mutant had 32%. The reversal of the charge did not change the $K_m$ for NAD$^+$ or aldehyde substantially. This was in contrast to what we found with E487K/Q (21), where the Lys mutant possessed a very high $K_m$ for NAD$^+$ while the Gln mutant had native like $K_m$ values. Neither Glu$^{487}$ or Lys$^{489}$ are completely conserved, but a negative and a positive charge, respectively, are always found at these positions.

We can now offer some structural arguments for the effects of the various mutants we investigated. Prior to this study, we reported that Cys$^{302}$ is the active site nucleophile (10) and Glu$^{368}$ is the general base (11). It is possible that a water attached to Glu$^{368}$ may abstract the proton from Cys$^{302}$ (3). We previously showed that mutations to Ser$^{74}$ drastically affected the $K_m$ for NAD$^+$, while it did not affect $K_m$ for NADH (12). Ser$^{74}$ is located near the dimer interface and is associated with residues 69 and 71 which make contact between the pair of dimers. Similarly, Glu$^{399}$ makes contact with arginine residues in its own subunit as well as in the other dimer pair (3). When this residue was mutated to a lysine to mimic what has been found in many Oriental people (21), the $K_m$ for NAD$^+$ increased and the specific activity decreased. We argued that disrupting the glutamate-arginine salt bonds was responsible for the observed effect. Lys$^{489}$ also binds to the opposed subunit in the dimer pair, capturing the C terminus of a helix between residues 436 and 445. Thus, its role appears to be in stabilizing subunit interactions (3).

Residue 384 (threonine) prefers to have a hydroxyl as the side chain in that the serine mutant is more native-like than is the alanine. This residue is located near the solvent surface but near a hydrophobic core that extends toward the coenzyme binding pocket. Potentially, of more importance is that the hydroxyl group of Thr$^{384}$ binds to the carbonyl back bone of Pro$^{383}$. This interaction appears to maintain the local structure of the conserved Pro$^{383}$ and Thr$^{384}$ residues found in all ALDHs. Mutation to residue 471 (serine), as mentioned above, affects the $K_m$ for NAD$^+$. It is located such that it interacts with residues 269 and 270. Conceivably a disruption of that interaction would alter the position of Glu$^{268}$, the conserved general base. This disruption could explain the decrease in specific activity. From the structure it appears that Glu$^{268}$ might have to move to accommodate the binding of NAD$^+$. The calculated $k_2$ term decreased 1500-fold in the S471A mutant and $k_{cat}$ increased 15-fold, consistent with the argument that an alteration near 268 could affect NAD$^+$ interactions with the enzyme for Ile$^{265}$ is in van der Waal contact with the nicotinamide ring.

Arg$^{84}$ is part of a long helix between residues 81 and 110 and interacts through a water molecule with the C-terminal Ser$^{200}$ in a different subunit. Furthermore, it serves to cap a helix in its own subunit by binding to the peptide carbonyls of residues 183 and 184. Removing the charge did not alter drastically the catalytic properties. A negative charge at this position affected $k_{cat}$ without increasing $K_m$ for either substrate. The Arg$^{84}$ containing helix is located near the subunit interface. Lower concentrations of R84E and K489E were found after expressing the mutants in E. coli. Mutation to these residues could have affected assembly. It is not apparent why lower levels of K192E were also found. This residue appears to be exposed to solvent and might not be expected to affect assembly or stability. It will be necessary to study the stability of the mutants to determine if the point mutations did actually affect assembly. Both residues 192 and 399 bind to ribose hydroxyl groups and appear to be the only conserved residues that directly interacts with the coenzyme. It is surprising that mutation to only one drastically alters NAD$^+$ binding. However, the nicotinamide ring appears to have to move during the catalytic process (3) and since Glu$^{399}$ is bound to the nicotinamide ribose, it may not be unexpected that this residue should be less important in the binding of NAD$^+$. The major affect of mutating Glu$^{399}$ is a change in the rate-limiting step, while mutating Lys$^{192}$ causes a change in both NAD$^+$ binding and the rate-limiting step. Apparently the proper anchoring of the NAD$^+$ is essential for the hydride transfer step.

Our laboratory has argued that since substrates with an electron withdrawing group, such as chloroacetaldehyde, are oxidized more rapidly than propionaldehyde, and a pre-steady state burst was observed, decylation, $k_1$ in Fig. 1, is rate-limiting for mitochondrial aldehyde dehydrogenase. Only with K192Q and E399Q were these properties not found. For those,
the rate of oxidation of chloroacetaldehyde was not greater than that of propionaldehyde, showing that the rate-limiting step did not involve the attack of the nucleophile, $k_3$ or $k_7$. Further, as no burst was found, $k_2$ and $k_6$ were eliminated as the rate-limiting step. Therefore, we conclude that the rate-limiting step was changed to hydride transfer ($k_9$) for these two mutants. Detailed studies presented in the following paper will show that there is a primary isotope effect on aldehyde oxidation, verifying the fact that hydride transfer became rate-limiting for K192Q and E399Q mutants (15).

The term $k_{cat}/K_{m(propionaldehyde)}$ is related to aldehyde interaction with the enzyme-NAD$^+$ complex (10).

$$k_{cat}/K_{m(propionaldehyde)} = k_9 k_3/(k_4 + k_3) \quad (\text{Eq. 1})$$

If it is assumed that the value of $k_3$ is small, due to the tight binding of aldehyde to enzyme-NAD$^+$ complex, then $k_{cat}/K_{m(propionaldehyde)}$ approximates to the value of $k_{cat}$. Mutations of native amino acids decreased this value by 2–40-fold.

Mitochondrial ALDH can hydrolyze activated esters such as p-nitrophenylacetate; presumably, both esterase and dehydrogenase reaction take place at the same active site (10–12, 28). All the mutants produced in this study still hydrolyzed the ester. The esterase reaction involves many of the same steps as the dehydrogenase reaction that the dehydrogenase reaction use, except it does not include the acylation step ($k_9$) (Fig. 1). The acylation step ($k_9$) is thought to be the rate-limiting step for the esterase reaction with horse (14) and human liver ALDH2 (11). This decreased esterase activity was found with the mutants that had a change in $k_5$ and those that produced a change in $k_3$ of the dehydrogenase reaction.

The formation of the tetrahedral intermediate in the esterase reaction should be similar to that of the hemiacetal formation in the dehydrogenase reaction. Decrease in the esterase activity, irrespective of which step was involved in the rate-limiting step of the dehydrogenase reaction, indicates that the mutation also affected the $k_3$ and $k_9$ steps, which are common to both esterase and dehydrogenase reaction (Reactions 1 and 2).

![Image](image-url)

**Reaction 1. Dehydrogenase reaction.**

$$E + SH + RCHO \rightleftharpoons ESC R \rightleftharpoons ESC R$$

$$| NAD^+ \rightleftharpoons OH \rightarrow NADH$$

**Reaction 2. Esterase reaction.**

$$O\text{SH} + RCONP \rightarrow \text{ESCONP} \rightarrow \text{ESC-R}$$

$$| NAD^+ \rightarrow O \rightarrow R \rightarrow NAD^+$$

A correlation ($r^2 \approx 0.74$) existed between the calculated $k_3$ values for dehydrogenase and the esterase velocity as shown in Fig. 3. The esterase activity of K192Q enzyme appeared to be higher than expected. This might be related to the fact that this mutant had higher activity with the aromatic substrates, as will be discussed in the accompanying paper (15). For the esterase reaction an additional, nonhydride transfer step ($k_9$) is required to form the acyl intermediate. Conceivably the mutations could affect this step as well as the $k_3$ step needed to form the tetrahedral intermediate.

This study showed that of the conserved residues with reactive side chains, other than Cys$^{92}$ and Glu$^{399}$, only Lys$^{192}$ and Glu$^{399}$ could be considered to be involved in the active site of the enzyme. The other residues appear to make long ranged contacts with residues which make it possible for

the catalytic residues to be properly aligned, analogous to Asp$^{122}$ making a salt bond with Ile$^{122}$ in chymotrypsin (29). The two ribose-binding residues were essential in catalysis in that the rate-limiting step for the enzyme changed when these were mutated. However, mutation of any completely or partially conserved residue appeared to affect more than one step in the catalytic reaction. Preliminary results on the mutational analysis of the conserved Glu$^{399}$ in fatty aldehyde dehydrogenase from luminescent bacteria (*Vibrio harveyi*) were reported to be similar to the corresponding Glu$^{399}$ in mitochondrial ALDH2 (30). A more detailed analysis of the precise involvement of the Lys$^{192}$ and Glu$^{399}$ will be presented in the following paper (15).

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