Involvement of Glutamate 399 and Lysine 192 in the Mechanism of Human Liver Mitochondrial Aldehyde Dehydrogenase

Li Ni, Saifuddin Sheikh, and Henry Weiner‡
From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153

(Received for publication, December 23, 1996, and in revised form, April 20, 1997)

Mutation to the conserved Glu399 or Lys192 caused the rate-limiting step of human liver mitochondrial aldehyde dehydrogenase (ALDH2) to change from deacylation to hydride transfer (Sheikh, S., Ni, L., Hurley, T. D., and Weiner, H. (1997) J. Biol. Chem. 272, 18817–18822). Here we further investigated the role of these two NAD+ ribose-binding residues. The E399Q/K/H/D and K192Q mutants had lower dehydrogenase activity when compared with the native enzyme. No pre-steady state burst of NADH formation was found with the E399Q/K and K192Q enzymes when propionaldehyde was used as the substrate; furthermore, each mutant oxidized chloroacetalddehyde slower than propionaldehyde, and a primary isotope effect was observed for each mutant when [2H]acetaldehyde was used as a substrate. However, no isotope effect was observed for each mutant when α-[2H]benzaldehyde was the substrate. A pre-steady state burst of NADH formation was observed for the E399Q/K and K192Q mutants with benzaldehyde, and p-nitrobenzaldehyde was oxidized faster than benzaldehyde. Hence, when aromatic aldehydes were used as substrates, the rate-limiting step remained deacylation for all these mutants. The rate-limiting step remained deacylation for the E399H/D mutants when either aliphatic or aromatic aldehydes were used as substrates. The K192Q mutant displayed a change in substrate specificity, with aromatic aldehydes becoming better substrates than aliphatic aldehydes.

Two conserved residues of mitochondrial aldehyde dehydrogenase (ALDH1) were found to bind the NAD+ ribose rings. Lys192 bound the adenine ribose, while Glu399 bound the nicotinamide ribose (1). When they were separately mutated to glutamine residues, the mutants were found to have a small isotope effect when the substrate was chloroacetalddehyde or benzaldehyde (1). When they were separately mutated to glutamine residues, the mutants were found to have a small isotope effect when the substrate was chloroacetalddehyde or benzaldehyde (1).

The K192Q mutant displayed a change in substrate specificity when either aromatic or aliphatic aldehydes were used as substrates. The E399Q mutant oxidized chloroacetalddehyde and benzaldehyde slower than propionaldehyde, and a pre-steady state burst of NADH formation was observed for the K192Q mutant when propionaldehyde was used as a substrate. However, no pre-steady state burst of NADH formation was observed with the E399Q/K and K192Q enzymes when propionaldehyde was used as the substrate; furthermore, each mutant oxidized chloroacetalddehyde slower than propionaldehyde, and a primary isotope effect was observed for each mutant when [2H]acetaldehyde was used as a substrate. However, no isotope effect was observed for each mutant when α-[2H]benzaldehyde was the substrate. A pre-steady state burst of NADH formation was observed for the E399Q/K and K192Q mutants with benzaldehyde, and p-nitrobenzaldehyde was oxidized faster than benzaldehyde. Hence, when aromatic aldehydes were used as substrates, the rate-limiting step remained deacylation for all these mutants. The rate-limiting step remained deacylation for the E399H/D mutants when either aliphatic or aromatic aldehydes were used as substrates. The K192Q mutant displayed a change in substrate specificity, with aromatic aldehydes becoming better substrates than aliphatic aldehydes.

This paper is available online at http://www.jbc.org

This work was supported in part by National Institutes of Health Grant AA05812. This is journal paper 15431 from the Purdue Agricultural Experiment Station. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, Purdue University, W. Lafayette, IN 47907-1153. Tel.: 765-494-1650; Fax: 765-494-7897; E-mail: weiner@biochem.purdue.edu.

The abbreviations used are: ALDH, aldehyde dehydrogenase; ALDH1, cytosolic aldehyde dehydrogenase; ALDH2, mitochondrial aldehyde dehydrogenase; ALDH3, microsomal aldehyde dehydrogenase; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; Vmax, the dehydrogenase activity of the enzyme when acetaldehyde or benzaldehyde was oxidized; Km, the dehydrogenase activity of the enzyme when [2H]acetaldehyde or α-[2H]benzaldehyde was oxidized.
Roles of Glu<sup>399</sup> and Lys<sup>192</sup> in ALDH Catalysis

The dehydrogenase activity assay was performed in the 100 mM sodium phosphate buffer (pH 7.4). The burst magnitude was expressed as the moles of NADH formation/mol of the tetrameric enzyme.

4 tables are included in the document which provide kinetic constants for the native and mutant forms of human liver mitochondrial aldehyde dehydrogenase (ALDH2). The tables compare the values for NAD<sup>+</sup> and propionaldehyde for the wild-type (Native) enzyme and several mutants including E399K, E399Q, E399H, E399D, and K192Q.

The burst magnitude at different pH values for the mutants K192Q, E399K, E399Q, and E399H is also provided, showing a decrease with increasing pH for the E399Q mutant.

The study concludes that the rate-limiting step for the mutants E399Q/K became hydride transfer (k<sub>c</sub>) as with the E399Q mutant.

Aldehydes Were Used as Substrates for the E399Q/K Mutants—It was shown that there was a pre-steady state burst of NADH formation with human liver mitochondrial ALDH; the burst magnitude was approximately 2 mol of NADH/mol of tetrameric enzyme (4). This implied that decylation (k<sub>p</sub>) or NADH dissociation (k<sub>a</sub>) was the rate-limiting step for the native ALDH2 enzyme (see Fig. 1 in Ref. 2). Other data proved that decylation (k<sub>p</sub>) was the rate-limiting step for the native ALDH2 enzyme (11–13).

Similar to what was found with the E399Q mutant (2), no pre-steady state burst of NADH formation was observed with the E399K enzyme when propionaldehyde was used as the substrate (Table II). Furthermore, the E399K mutant oxidized chloroacetaldehyde slower than propionaldehyde (Table III), indicating that the rate-limiting step changed. Since the chloro group makes oxidation more difficult to occur, it implied that the rate-limiting step for the E399K became hydride transfer (k<sub>c</sub>), as with the E399Q mutant.

To study the rate-limiting step for the mutants E399Q/K, the primary isotope effect on the aldehyde oxidation was determined (Table IV). No primary isotope effect was found for the native ALDH2 enzyme with either α-[<sup>2</sup>H]benzaldehyde or [<sup>14</sup>CO]acetaldehyde, consistent with hydride transfer not being the rate-limiting step for the native enzyme, and also consistent with what we found for the horse liver mitochondrial ALDH (11, 12). A V<sub>i</sub>/V<sub>p</sub> of 2.5, however, was observed for the E399Q and E399K mutant enzymes, when [<sup>14</sup>CO]acetaldehyde was used as the substrate, consistent with hydride transfer (k<sub>p</sub>) being at least a partial rate-limiting step.

In contrast to what we found with [<sup>14</sup>CO]acetaldehyde, no primary isotope effect was observed when α-[<sup>2</sup>H]benzaldehyde was used as the substrate. This indicated that the rate-limiting step was not hydride transfer (k<sub>c</sub>) when benzaldehyde was used as the substrate for the E399Q/K mutants. This was verified by finding a pre-steady state burst when benzaldehyde was the substrate for the E399Q/K mutant enzymes (Table II). Furthermore, the k<sub>c</sub> of aromatic aldehyde oxidation was related to the structure of the substrate. p-Nitrobenzaldehyde, an aromatic aldehyde having an electron-withdrawing group, was oxidized much faster than those lacking the group (Table V). This indicated that the rate-limiting step for aromatic substrates was still deacylation (k<sub>a</sub>) for the E399Q/K mutant enzymes, as with the native ALDH2 enzyme.

Rate-limiting Step Changed for the K192Q Mutant when Aliphatic and Aromatic Aldehydes Were Used as Substrates—No pre-steady state burst of NADH formation was found with the K192Q enzyme when propionaldehyde was used as the substrate (Table II). We showed that the K192Q mutant oxidized chloroacetaldehyde slower than propionaldehyde (2), indicating that the rate-limiting step changed. Since the chloro group makes oxidation more difficult to occur, it implied that
the rate-limiting step became hydride transfer ($k_b$), which was now verified by the pre-steady state burst data (Table II).

A $V_{p}/V_{a}$ of 2.0 was observed for the K192Q mutant enzyme (Table IV), when $[^2H]$acetaldehyde was used as the substrate, consistent with hydride transfer ($k_b$) being involved in the rate-limiting step. In contrast, when $\alpha$-[^2H]benzaldehyde was used as the substrate for the K192Q mutant, no primary isotope effect was observed. This indicated that the rate-limiting step was not hydride transfer ($k_b$) when benzaldehyde was used as the substrate. Only a small pre-steady state burst of NADH formation was observed during the oxidation of benzaldehyde by the K192Q mutant enzyme (Table II). The $k_{cat}$ of aromatic aldehyde oxidation was related to the structure of the substrate. p-Nitrobenzaldehyde was oxidized much faster than the others (Table V). This indicates that the rate-limiting step was not hydride transfer ($k_b$) for the K192Q mutant when aromatic aldehydes were used as substrates. Thus, the rate-limiting step differed for the K192Q mutant when aliphatic or aromatic aldehydes were oxidized.

The E399H/D Mutants of Human Mitochondrial ALDH2—
The E399H/D mutants oxidized chloroacetaldehyde faster than propionaldehyde (Table III). No isotope effects were found with the E399H/D mutants when using either $\alpha$-[2H]benzaldehyde or [2H]acetaldehyde as substrates, showing that hydride transfer was not the rate-limiting step for the E399H/D mutants when either aliphatic or aromatic aldehydes were used as substrates (Table IV). Furthermore, a pre-steady state burst of NADH formation was observed for the E399H/D mutants during the oxidation of both propionaldehyde and benzaldehyde (Table II), and the $k_{cat}$ was a function of substrates (Table V). These results indicated that the rate-limiting step for the E399H/D mutants was still deacylation ($k_d$).

E399H had many properties that mimic those of the native enzyme. The $K_m$ for NAD$^+$ increased 8-fold in the E399H mutant, and the $k_{cat}$ decreased to 5% of the native enzyme. Although the $k_{cat}$ of E399H was lower than the $k_{cat}$ of E399Q, the rate-limiting step remained deacylation ($k_d$) for E399H.

Aspartate could replace glutamate at position 399. Both $K_m$ for NAD$^+$ and $k_{cat}$ were native-like. This indicated that a negative charge was important for the proper function of the nicotinamide ribose-binding residue at position 399.

Effect of Magnesium Ions on the $k_{cat}$ of the Native, Glu$^{399}$, and Lys$^{192}$ Mutant Forms of ALDH2—It has been reported that the mammalian liver mitochondrial ALDHs can be activated by magnesium ions (9, 14–17). Native ALDH2 and the E399H/D enzymes could be activated approximately 2-fold by Mg$^{2+}$ ions when using either propionaldehyde or benzaldehyde as the substrate. In contrast, the E399Q/K and K192Q mutants were not activated by Mg$^{2+}$ ions when propionaldehyde was used as the substrate. They could be activated 2–5-fold by Mg$^{2+}$ ions when benzaldehyde was used as the substrate.

## Discussion

The structure of mitochondrial ALDH reveals that Lys$^{192}$ binds to the adenosine ribose and that Glu$^{399}$ binds to the nicotinamide ribose. When the Mg$^{2+}$ ions were present at low concentration in the crystals, a disordering of the nicotinamide portion of the coenzyme was observed (1). ALDH is active in the absence of Mg$^{2+}$ ions, but the presence of Mg$^{2+}$ ions causes the enzyme to have an increased specific activity and the pre-steady state burst magnitude of NADH formation, at least for the enzyme from horse (14) and rat (9), increases from 2 mol of NADH/mol of enzyme to 4. Here we found that only the E399H or E399D mutants were affected by the addition of Mg$^{2+}$ ions, analogous to what is found with the native human enzyme. These two mutants had the most native-like behavior in that the rate-limiting step remained deacylation ($k_d$).

A glutamate residue can replace the function of the lysine at position 192; the $K_m$ for NAD$^+$ and the rate-limiting step for the K192E were native-like. The specific activity of K192E was depressed, compared with the native enzyme or even the glutamine mutation. Since residue 192 does not appear to be in direct contact with any residue in the substrate binding domain or near other components of the active site, as shown in Fig. 1 of the accompanying paper (2), we can only assume that the effect on $k_{cat}$ was due to a transitional state stabilization such that hydride transfer is not functioning efficiently when the adenosine ribose is not bound by the lysine residue. For the esterase reaction of the enzyme, where hydride transfer is not involved, the K192Q mutant was shown to have 50% the activity of the native enzyme, compared with 19% of the dehydrogenase activity (2). Finding that the esterase activity was diminished shows that residue 192 does indeed influence the catalytic site even in the absence of NAD$^+$.

In the accompanying paper (2), we proposed that mutations to the two ribose-binding residues, Lys$^{192}$ and Glu$^{399}$, caused the rate-limiting step to change to hydride transfer. Here we show that when $[^2H]$acetaldehyde was the substrate, a primary isotope effect was found with K192Q and E399Q (Table IV). Unlike native enzyme, where a pre-steady state burst of NADH was found, neither of the two mutants produced a burst, consistent with the other data presented, which indicated that the rate-limiting step did change. A most unexpected result was found when $[^2H]\alpha$-benzaldehyde was employed as the substrate. No primary isotope effect was found for the 192 and 399 mutants. This implies that the rate-limiting step with aromatic aldehydes remained deacylation. Finding that there was a pre-steady state burst and that $p$-nitrobenzaldehyde was oxidized more rapidly than was benzaldehyde is consistent with this conclusion.

It is not possible to unequivocally explain why for the two mutant enzymes did the rate-limiting step become hydride transfer with aliphatic aldehydes but remain deacylation with aromatic aldehydes. Cys$^{302}$ is located 7.2 Å from Glu$^{399}$ and 17 Å from Lys$^{192}$. Thus, it is not possible for these two residues to interact directly with the substrate being oxidized, but their presence is clearly transmitted to the region surrounding the substrate pocket. Hydride transfer from aliphatic substrates appears to be more sensitive to the precise positioning of NAD$^+$, which is held in place by Lys$^{192}$ and Glu$^{399}$. The transition state for hydride transfer from aromatic aldehydes could be partially stabilized by the presence of the II electron cloud. If so, it could explain why the rate-limiting step differs.

The E399K mutant was prepared since a lysine functions at position 192. Unlike with E399Q, the $K_m$ for NAD$^+$ increased 12-fold and the $k_{cat}$ decreased to 0.5% of the native enzyme. Thus, the ligand to the nicotinamide ribose can not be lysine. Aspartate could function well at that position; the E399D mu-
Roles of Glu^{399} and Lys^{192} in ALDH Catalysis

### Table IV

The primary ^2H isotope effect on the native and mutant forms of human liver mitochondrial aldehyde dehydrogenase (ALDH2)

| Enzyme | Acetaldehyde | Benzylaldehyde |
|--------|--------------|----------------|
|        | V_H / V_D | V_H / V_D | V_H / V_D |
| Native | 980         | 4.8           | 170         |
| E399K  | 110         | 51            | 170         |
| E399Q  | 490         | 90            | 50          |
| E399H  | 490         | 90            | 50          |
| K192Q  | 190         | 51            | 170         |

*V_H* refers to the dehydrogenase activity (n mol/min/mg protein) when acetaldehyde or benzylaldehyde was used as the substrate; *V_D* refers to the dehydrogenase activity when [^2H]acetaldehyde or α[^2H]benzylaldehyde was used as the substrate.

### Table V

The dehydrogenase activities for the native and mutant forms of human liver mitochondrial aldehyde dehydrogenase (ALDH2) when different aldehydes were used as substrates

| Substrate       | Native | E399K | E399Q | E399H | E399D | K192Q |
|-----------------|--------|-------|-------|-------|-------|-------|
| Acetaldehyde    | 980    | 4.8   | 110   | 51    | 490   | 190   |
| Propionaldehyde | 870    | 4.3   | 92    | 44    | 440   | 160   |
| Chloroacetaldehyde | 3370   | 3.0   | 65    | 57    | 1610  | 75    |
| Benzaldehyde    | 170    | 19    | 50    | 66    | 71    | 170   |
| p-Nitrobenzaldehyde | 430    | 91    | 270   | 260   | 360   | 1490  |
| p-Metroxybenzaldehyde | 500    | 4.8   | 28    | 15    | 36    | 56    |

The unit of activity is n mol/min/mg protein.

### Notes and Discussion

**Roles of Glu^{399} and Lys^{192} in ALDH Catalysis**

The primary ^2H isotope effect on the native and mutant forms of human liver mitochondrial aldehyde dehydrogenase (ALDH2) is studied. The table shows the dehydrogenase activities for the native and mutant forms of human liver mitochondrial aldehyde dehydrogenase (ALDH2) when different aldehydes were used as substrates.

**References**

1. Steinmetz, C. G., Xie, P.-G., Weiner, H., and Hurley, T. D. (1997) *Structure* 5, 701–711
2. Sheikh, S., Ni, L., Hurley, T. D., and Weiner, H. (1997) *J. Biol. Chem.* 272, 18817–18822
3. Farrés, J., Wang, T. T. Y., Cunningham, S. J., and Weiner, H. (1995) *Biochemistry* 34, 2592–2598
4. Wang, X.-P., and Weiner, H. (1995) *Biochemistry* 34, 237–243
5. Studier, F. W., and Moffat, B. A. (1986) *J. Mol. Biol.* 189, 113–130
6. Zheng, C.-F., Wang, T. T. Y., and Weiner, H. (1993) *Alcoholism Clin. Exp. Res.* 17, 828–831
7. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463–5467
8. Jeng, J., and Weiner, H. (1991) *Arch. Biochem. Biophys.* 289, 214–222
9. Farrés, J., Wang, X.-P., Takahashi, K., Cunningham, S. J., Wang, T. T., and Weiner, H. (1984) *J. Biol. Chem.* 269, 13854–13860
10. Ghenbot, G., and Weiner, H. (1992) *Protein Exp. Purif.* 3, 470–478
11. Feldman, R. I., and Weiner, H. (1972) *J. Biol. Chem.* 247, 267–272
12. Feldman, R. I., and Weiner, H. (1972) *J. Biol. Chem.* 247, 260–266
13. Klyosov, A. A. (1996) *Biochemistry* 35, 4457–4467
14. Takahashi, K., and Weiner, H. (1980) *Biochemistry* 19, 4922–4926
15. Vallari, R. C., and Pietruszko, R. (1984) *J. Biol. Chem.* 259, 4927–4933
16. Guan, K.-L., Pak, Y. K., Tu, G.-C., Cao, Q.-N., and Weiner, H. (1988) *Alcoholism Clin. Exp. Res.* 12, 713–719
17. Takahashi, K., and Weiner, H. (1981) *Biochemistry* 20, 2729–2726

**Additional Points**

Addition of Mg^{2+} ions increases the activity of the mammalian ALDH2B by approximately 2-fold (9, 14–17). The stoichiometry of NADH binding remained 2 for the human ALDH2 in the presence of Mg^{2+} ions (data not shown), but it was increased to 4 for horse, beef, and rat ALDH2 (9, 17, 18). This suggested that for the human ALDH2 Mg^{2+} ions affected the rate-limiting step (k_f), while it increased the number of active sites for the others. Consistent with the statement is the fact that Mg^{2+} ions only increased the activity of the E399Q/K and K192Q mutants when aromatic aldehydes were oxidized, where the rate-limiting step was still k_f.

Between the data presented in this and the accompanying paper (2), we show that, although many residues were completely conserved in the ALDH family of enzymes, only those at position 302 and 268 appear completely essential while those at 399 and 192 are important for hydride transfer. The first two are involved chemically, where the later most likely are involved in stabilizing the transition state, resulting from their binding to the ribose ring in NAD^+.

**Conclusions**

The specific activity when [^2H]acetaldehyde or α[^2H]benzylaldehyde was used as the substrate.