Involvement of Glutamate 268 in the Active Site of Human Liver Mitochondrial (Class 2) Aldehyde Dehydrogenase As Probed by Site-Directed Mutagenesis†

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Received June 16, 1994; Revised Manuscript Received October 11, 1994®

ABSTRACT: On the basis of chemical modification studies, it was postulated that glutamate 268 was a component of the active site of liver aldehyde dehydrogenase [Abriola, D. P., Fields, R., MacKerell, A. D., Jr., & Pietruszko, R. (1987) *Biochemistry 26,* 5679−5684]. To study its role, the residue in human liver mitochondrial (class 2) aldehyde dehydrogenase was mutated to an aspartate, a glutamine, or a lysine, and the enzyme was expressed in *Escherichia coli.* The mutations did not affect the K_m values for NAD or propionaldehyde, but grossly affected the catalytic activity of the enzymes when compared to recombinantly expressed native enzyme; the mutant enzymes had less that 0.4% of the specific activity of the recombinantly expressed native aldehyde dehydrogenase. The mutations also caused a long lag phase to occur prior to the steady state phase of the reaction. The activity of the mutant enzymes could not be restored by the addition of general bases such as sodium acetate, sodium formate, or imidazole. The K_d for NADH was essentially identical for the E268Q mutant and native enzyme. The three mutant forms of the enzyme possessed less than 0.8% of the esterolytic activity of the recombinantly expressed native enzyme. Pre-steady state analysis showed that there was no burst of NADH formation in the dehydrogenase reaction or of *p*-nitrophenol formation in the esterase reaction. This can be interpreted as implying that glutamate 268 may function as a general base necessary for the initial activation of the essential cysteine residue (302), rather than being involved in only the deacylation or hydride transfer step. Alternatively, glutamate 268 could function as a component of a charge relay triad necessary to activate the nucleophilic residue. Furthermore, it appears that esterase and dehydrogenase require the same active site components, for both the dehydrogenase activity and esterase activity were essentially abolished when glutamate 268 was changed to another residue.

In spite of the fact that mammalian aldehyde dehydrogenase (ALDH)[1] was first purified to homogeneity in the early 1970s, all of the components of the active site have not been unequivocally identified. The enzyme appears to follow a mechanism similar to that catalyzed by glyceraldehyde-3-phosphate dehydrogenase (Feldman & Weiner, 1972; Weiner, 1979). That is, an active site nucleophile attacks the aldehyde to form a thiohemiacetal intermediate, which is then oxidized to form an acyl enzyme. Site-directed mutagenesis analysis verified the earlier conclusion that cysteine 302 is the active site nucleophile (Farrés *et al.*, 1995) and not serine 74 (Rout & Weiner, 1994) as proposed by Loomes *et al.* (1990) or cysteine 49 as we concluded from chemical modification studies (Tu & Weiner, 1988a). After the thioacyl intermediate is formed, it must be hydrolyzed, presumably by the action of general base catalysis. Mutational analysis showed that the highly conserved histidine residues in the enzyme were not functioning as the general base (Zheng & Weiner, 1993). Thus, a different residue would be necessary to carry out this aspect of the catalytic process.

Chemical modification of human liver cytosolic (class 1) and mitochondrial (class 2) ALDH with bromoacetophenone caused the inactivation of the enzyme and the loss of both cysteine 302 and glutamate 268 (Abriola *et al.*, 1987, 1990; Pietruszko *et al.*, 1991, 1993). These investigators proposed that E268 functioned at the active site as the possible general base. Later it was found that all ALDHs had a conserved glutamate at position 268, supporting the suggestion that the residue could indeed be functioning as a component in the active site of the enzyme (Hempel *et al.*, 1993).

The cDNA coding for human liver mitochondrial ALDH (class 2) has been expressed in our laboratory (Zheng *et al.*, 1993). The glutamate residue at position 268 was mutated to other residues in order to investigate its role in catalysis. In this study, we will show that E268 in human liver class 2 ALDH is not involved in coenzyme binding, as is E487 (Farrés *et al.*, 1994), but that it may function in catalysis as a general base or as part of a triad in a charged relay necessary for the initial activation of cysteine 302.

EXPERIMENTAL PROCEDURES

Materials. NAD and NADH were purchased from Sigma Chemical Company; the Sequenase Version 2.0 kit and phosphatase were obtained from United States Biochemical Corp.; *Bam*HI and *Xba*I were from New England Biolabs; propionaldehyde and *p*-nitrophenyl acetate were from Aldrich Chemical Company, Inc.; *Xma*I, the Magic Minipreps

† This work was supported in part by Grant AAO5812 from the National Institutes of Health. This is Journal Paper 14266 from the Purdue University Agricultural Experiment Station.

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® Abstract published in *Advance ACS Abstracts,* December 1, 1994.

[1] Abbreviations: ALDH, aldehyde dehydrogenase; IEF, isoelectric focusing; HAP, *p*-hydroxyacetophenone; E268X, glutamate 268 mutated to another residue.

0006-2960/95/0434-237$09.00/0

DNA purification system, and T4 DNA ligase were from Promega Corp; alkaline phosphatase-conjugated goat anti-rabbit IgG, IEF standards, and the Muta-Gene *in vitro* mutagenesis kit were from Bio-Rad Laboratories, Inc.; the Geneclean kit was from Biol 101, Inc.; [α-^{35}S] dATP was from Amersham Corp.; agarose IEF and Pharmalyte were from Phamacia Biotech Inc.; nitrocellulose membranes were from Schleicher and Schuell Inc.

Plasmid, Phage, and Bacterial Strains. *Escherichia coli* CJ 236 (Bio-Rad) or MV 1190 (Bio-Rad) served as hosts for M13 phage. Native and mutant ALDH cDNAs were cloned into the pT7-7 expression vector, which is a derivative of pT7-1 described by Tabor and Richardson (1985), and expressed in *E. coli* strain BL21 (DE3) pLysS (Studier & Moffatt, 1986; Studier *et al.*, 1990) as we previously reported (Zheng *et al.*, 1993).

DNA Sequencing. DNA sequencing was performed with the Sequenase Version 2.0 kit by following the protocols provided by the manufacturer.

Immunoblotting. For determining the presence of ALDH during the purifications, 5 μL of each fraction, E268D, E268Q, and E268K, was pipetted onto nitrocellulose membranes for immunoblotting as previously described (Farrés *et al.*, 1994). The nitrocellulose membranes were subjected to laser densitometer scanning to estimate the amount of enzyme added to the membrane.

Cloning of cDNA Coding for ALDH into the M13mp18 Vector. cDNA for human liver class 2 ALDH was cloned into the *Nde*I and *Bam*HI sites of pT7-7 (Zheng *et al.*, 1993). After restriction enzyme digestions, the 1.5 kb *Xba*I/*Bam*HI fragment of pT7-7−ALDH was cloned into the *Xba*I/*Bam*HI site of M13mp18.

Site-Directed Mutagenesis. The GAG codon for glutamate 268 was changed to GAT, CAG, and AAG to construct E268D, E268Q, and E268K, respectively. Site-directed mutagenesis was performed following instructions from Bio-Rad. All mutations were confirmed by sequencing the mutant fragments.

Construction of Expression Vectors. Expression vectors for E268D, E268Q, and E268K were constructed by replacing the native *Xma*I/*Xma*I fragment of pT7-7−ALDH with the corresponding *Xma*I/*Xma*I fragment from the replicative forms of the mutant M13mp18 derivatives.

Expression of Native and Mutant ALDHs. Native and mutant forms of human liver mitochondrial (class 2) ALDH were expressed as previously described (Zheng *et al.*, 1993; Farrés *et al.*, 1994).

Purification of ALDH. Native or mutant enzymes were purified through DEAE and HAP chromatography (Farrés *et al.*, 1994; Rout & Weiner, 1994). During purification, the activity of recombinantly expressed ALDH was monitored at pH 7.4. For mutant enzymes E268D, E268Q, and E268K, dot blotting was used to determine the fractions containing the mutant enzymes. Purity was checked by SDS−PAGE staining with Coomassie Blue.

Fluorescence Assay for the Dehydrogenase Activity. Dehydrogenase activity assays were performed in 100 mM sodium phosphate (pH 7.4) at 25 °C by monitoring the formation of NADH 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 0.8 mM NAD and 14 μM propionaldehyde.

Spectrophotometric Assay for Esterase Activity. Esterase activity assays were performed at 400 nm using 150 μM *p*-nitrophenyl acetate as the substrate in 100 mM sodium phosphate (pH 7.4). A molar extinction coefficient of 18.3 $\times$ 10^3 at 400 nm for *p*-nitrophenolate and a pK_a of 7.1 for *p*-nitrophenol were used to calculate the rates in terms of micromoles of *p*-nitrophenyl ester hydrolyzed per minute (Sidhu & Blair, 1975). Activity values were corrected by subtracting the rate of the nonenzymic hydrolysis of *p*-nitrophenyl acetate.

Molecular Weight Determination. The molecular weights of the E268Q and E268K mutant enzymes were estimated by HPLC with a TSK-G3000Sw column, in 150 mM sodium phosphate and 0.05% sodium azide (pH 7.0), using recombinantly expressed native enzyme as a standard (Farrés *et al.*, 1994).

Pre-Steady State Burst of NADH Formation. The pre-steady state burst magnitude of NADH formation was measured on a Hitachi 2000 Fluorescence Spectrophotometer (Farrés *et al.*, 1994; Rout & Weiner, 1994). Enzyme and NAD were incubated in 100 mM sodium phosphate (pH 7.4) to establish a fluorescence base line. At a time called zero, propionaldehyde was added to initiate the reaction. The extrapolated line intersecting at time zero gave the magnitude of the burst of NADH formation. Concentrations of NAD were 1 and 5 mM for native and mutant enzymes, respectively. The propionaldehyde concentration was 140 μM. By calibrating the fluorometer with NADH, it was possible to calculate the moles of NADH produced prior to the steady state rate of NADH formation (Weiner *et al.*, 1976).

Pre-Steady State Burst of Ester Hydrolysis. The pre-steady state burst magnitude of ester hydrolysis was measured with a Gilford spectrophotometer. At a time called zero, 300 μM *p*-nitrophenyl acetate was added to the enzyme in buffer to initiate the reaction. The extrapolated line intersecting at time zero gives the magnitude of the burst of ester hydrolysis (Weiner *et al.*, 1976).

Protein Concentration. Protein concentration was determined with the Bio-Rad protein determination kit, using bovine serum albumin as a standard.

Slab Gel Isoelectric Focusing. Analytical isoelectric focusing was performed in a 1% agarose slab gel using Pharmalyte, pH 4−6.5. Focused samples were detected by protein staining with Coomassie Blue (Ghenbot & Weiner, 1992).

NADH Binding. The binding of NADH to the native and E268Q enzymes was performed by titrating the enzyme with NADH and measuring the emission at 450 nm with excitation at 340 nm (Takahashi *et al.*, 1980; Rout & Weiner, 1994).

RESULTS

Expression and Purification of Native and Mutant Enzymes. Recombinantly expressed native and E268 mutant forms of human liver class 2 aldehyde dehydrogenase were purified to homogeneity by established methods (Ghenbot & Weiner, 1992; Farrés *et al.*, 1994; Rout & Weiner, 1994), as judged by SDS−PAGE followed by staining with Coomassie Blue. The presence of an immunological cross-reacting protein was verified by Western blotting with

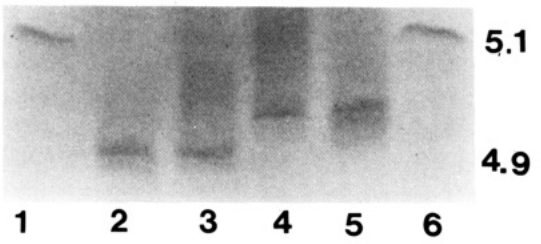


FIGURE 1: Isoelectric focusing of recombinantly expressed native or mutant forms of human liver class 2 mitochondrial ALDH: lanes 1 and 6, IEF marker, pH 5.1; lane 2, native ALDH; lane 3, E268D; lane 4, E268Q; lane 5, E268K. The p*I*'s for the native enzyme and E268D were 4.9 and those for E268Q and E268K were 5.0.

antibodies prepared against beef liver (class 2) mitochondrial aldehyde dehydrogenase. It was estimated that all mutant enzymes were expressed at levels comparable to that of the native enzyme, as judged by SDS−PAGE and Western blotting. The mutant enzymes were tetramers having the same molecular weights as that of the native enzyme (216 800) (Hempel *et al.*, 1985). E268D, E268Q, and E268K exhibited no detectable catalytic activity in crude cell extracts using 0.8 mM NAD and 140 μM propionaldehyde in 100 mM sodium phosphate (pH 7.4). It was possible to assay for the presence of native enzyme in crude homogenates under those conditions. The isoelectric point of the E268D enzyme was 4.9, identical to that reported for the native enzyme. Removal of the negative charge caused an increase in the p*I* value; it was 5.0 for E268Q and slightly greater for E268K, as shown in Figure 1. The fact that the p*I* increased when E268 was mutated is consistent with the suggestion that amino acid residue 268 is accessible to solvents (Loomes & Jörnvall, 1991).

Substrate Binding Sites of the Mutant Enzymes Were Intact. It previously was shown that recombinantly expressed human liver aldehyde dehydrogenase bound to a 4-hydroxyacetophenone affinity column, a competitive inhibitor matrix (Ghenbot & Weiner, 1992). All three 268 mutant enzymes bound to the affinity column and were eluted with the same concentration of HAP, indicating that the substrate binding site was intact.

NADH Binding. It is known that the fluorescence of NADH increases when bound to ALDH (Takahashi *et al.*, 1980; Ambroziak *et al.*, 1989; Rout & Weiner, 1994). The maximum emission of NADH in the NADH−enzyme complex had a blue shift compared to free NADH when excited at 340 nm.

The fraction (α) of NADH bound to ALDH during the titration was determined as before (Takahashi *et al.*, 1980), on the basis of the equations developed by Laurence (1952):

$$\alpha = \frac{(F/F_f) - 1}{\lambda - 1}$$

where F and F_f are the measured fluorescence intensities of the NADH−enzyme complex and free NADH, respectively, at the same coenzyme concentration, and λ is the enhancement factor that represents the ratio of the fluorescence intensity of bound NADH to that of free NADH.

To determine λ, NADH was added to a fixed amount of enzyme until no further enhancement in the fluorescence of added NADH was obtained (Figure 2). The value was calculated from the y-intercept, which is $(\lambda - 1)$(slope)[E]. The value of λ was calculated to be 4.9 for native and 4.5 for E268Q. The binding data were graphed as Scatchard plots, as shown in Figure 2. It could be estimated that about 1.5 and 2 mol of NADH bound per mole of tetrameric native and E268Q, respectively. The values of K_d for NADH dissociation were also similar for native and E268Q enzymes, 4.6 and 6.2 μM, respectively.

Assay of Dehydrogenase Activity. A lag, prior to the steady state portion of the reaction, was observed when assaying the pure, recombinantly expressed native enzyme. A lag has often been observed when analyzing the native enzyme, especially the one from horse liver mitochondria (K. Takahashi and H. Weiner, unpublished observations). A lag time from several seconds up to 1 min is typically found. When the E268Q mutant was analyzed, a 10 min lag was found, as shown in Figure 3. From the steady state portion of the curve, it could be calculated that the glutamine mutant possessed just 0.02% of the specific activity of the expressed native enzyme. The aspartate and lysine mutants behaved in a similar manner. The dehydrogenase activities of E268D and E268K were found to be just 0.4% and 0.2%, respectively, compared to that of native enzyme when the substrate concentrations were 2 mM NAD and 140 μM propionaldehyde (Table 1).

The K_m values for NAD and for propionaldehyde were determined for the recombinantly expressed native enzyme and the three mutants. Even though the specific activities of the mutant enzymes were less than 0.4% of that of the native enzyme, the values for K_m's were within a factor of about 2 of the native enzyme. The data are tabulated in Table 1.

Assay of Esterase Activity. It was shown that horse and human liver aldehyde dehydrogenases exhibit esterase activity in the absence and presence of coenzyme (Feldman *et al.*, 1972; Sidhu & Blair, 1975; Vallari & Pietruszko, 1981; Pietruszko *et al.*, 1993). All three mutant forms of the enzyme possessed limited esterolytic activity (less than 0.8%) when compared to that of the recombinantly expressed native enzyme. NAD was found to enhance the activity 2-fold for each mutant, as presented in Table 2.

Does Glutamate 268 Function in the Deacylation or Acylation Step? It has been assumed that a general base functions in the deacylation of the enzyme thioester intermediate by extracting a proton from water. The hydroxyl group has been postulated to attack the carbonyl carbon of the thioester to hydrolyze the acyl intermediate (Weiner, 1979). It is not known whether a general base is necessary for the formation of the initial hemiacetal intermediate. If glutamate 268 functioned as a general base only in the deacylation step, the enzyme lacking this residue should still be able to produce 1 mol of NADH per active site prior to the slow deacylation step. To test for this, the pre-steady state burst of NADH was measured. A burst was found with recombinantly expressed native enzyme, as we previously showed to occur with different liver ALDHs. Only 1.7 mol of NADH was produced per mole of recombinant native enzyme, indicating that recombinant native enzyme possessed half of the site reactivity, as we have previously demonstrated (Weiner *et al.*, 1976; Takahashi & Weiner, 1980; Farrés *et al.*, 1994; Rout & Weiner, 1994). In contrast to what we found with native enzyme, no burst of NADH formation was observed with the mutant enzyme forms. This observation

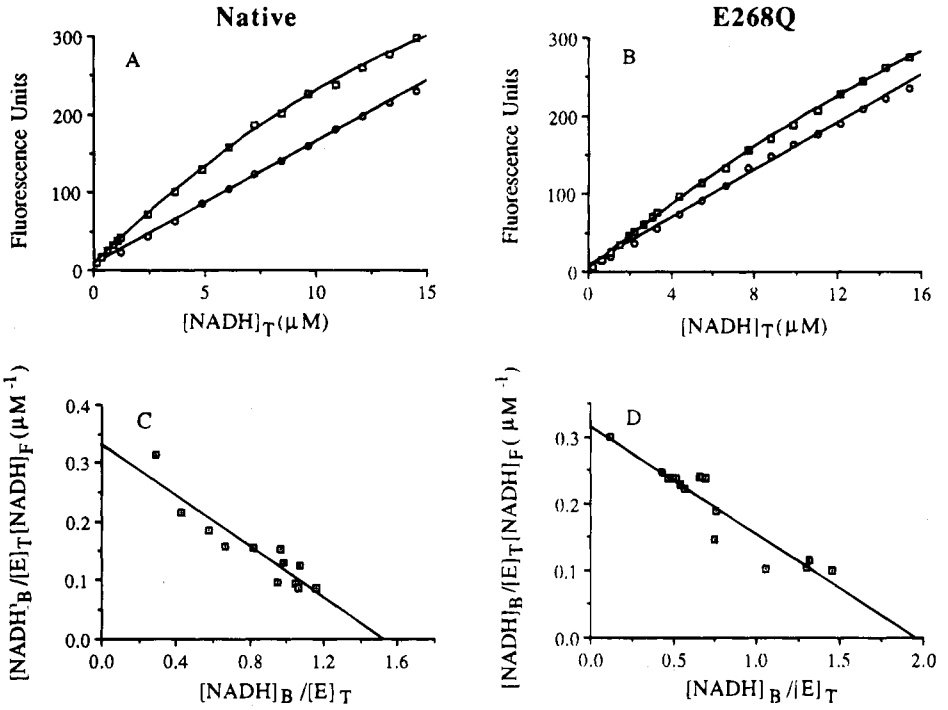


FIGURE 2: Fluorescence titration of NADH binding to recombinantly expressed aldehyde dehydrogenases. (A) Fluorescence titration for recombinant native enzyme. The enzyme concentration was between 0.95 and 0.94 μM. (B) Fluorescence titration for E268Q. The enzyme concentration was between 0.52 and 0.50 μM. Fluorescence was in the absence of enzyme (○) and in its presence (□). (C and D) Scatchard plots corresponding to the titration data for native enzyme (C) and E268Q (D), respectively.

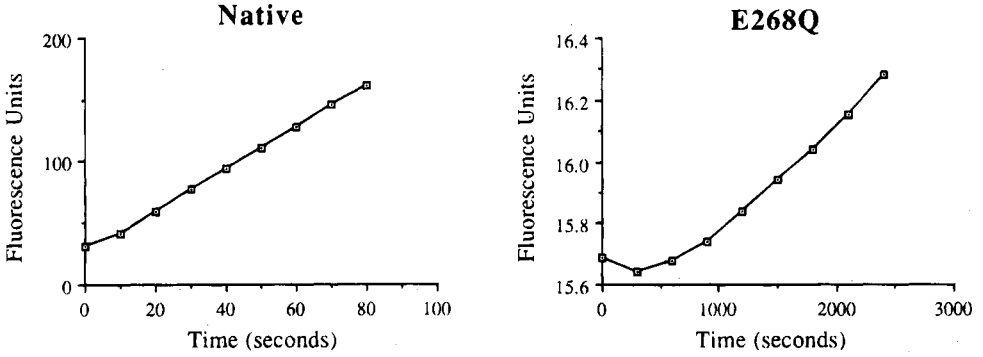


FIGURE 3: Lag phase prior to the steady state phase of the dehydrogenase reaction. Aldehyde oxidation was assayed in 100 mM sodium phosphate with a Hitachi F-2000 fluorescence spectrophotometer and is reported in arbitrary units. The concentration of NAD was 1 mM and that of propionaldehyde was 140 μM. The amount of native enzyme was 5.5 μg/mL and that of E268Q was 22 μg/mL.

Table 1: Dehydrogenase Activities for Native and Position 268 Mutant Forms of Human Liver Mitochondrial Class 2 Aldehyde Dehydrogenase[a]

enzyme	K_m (μM) NAD	K_m (μM) propionaldehyde	k_{cat} (min^{-1})	percentage of k_{cat}
native	28	0.60	205	100
E268D	31	0.24	0.82	0.4
E268Q	22	0.62	0.04	0.02
E268K	55	1.6	0.41	0.2

[a] Assays were performed in 100 mM sodium phosphate (pH 7.4).

Table 2: Esterase Activities of Native and Mutant Forms of Human Liver Mitochondrial Class 2 Aldehyde Dehydrogenase in the Presence and Absence of 2 mM NAD

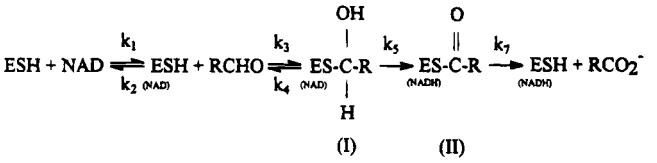
	−NAD		+NAD	
enzyme	activity[a]	percentage	activity	percentage
native	190	100	828	100
E268D	1.5	0.79	3.5	0.42
E268Q	1.3	0.68	2.6	0.31
E268K	1.4	0.74	2.3	0.28

[a] The unit for activity is nanomoles/minute/milligram. Assays were performed in 100 mM sodium phosphate (pH 7.4) and 150 μM *p*-nitrophenyl acetate.

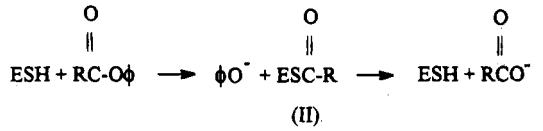
leads us to suggest that glutamate 268 could affect the initial formation of the intermediate hemiacetal (I, k_3) or its oxidation (k_5), and not just the hydrolysis (k_7) of the acyl intermediate (II) formed after the oxidation of the hemiacetal. Ester hydrolysis involves the attack of cysteine to form an acyl intermediate (II) without invoking oxidation.

If glutamate 268 functioned in the oxidation of the bound aldehyde (I) in Scheme 1 and/or in the hydrolysis of the acyl intermediate (II) (Schemes 1 and 2 (ϕ in Scheme 2 represents nitrophenol)), and not as a general base necessary to activate cysteine, one would expect to find an initial burst of nitrophenol in the esterase reaction because the first step, acylation, could still occur. However, no nitrophenol was produced in the pre-steady state with E268D. This leads us to conclude that glutamate 268 must be involved in the initial activation of cysteine 302 necessary to covalently bind the

Scheme 1



Scheme 2



substrate to the enzyme. Thus, it can be concluded that glutamate 268 may function in the catalytic process by acting as a general base (III) or by being part of a triad (IV) necessary to activate cysteine 302, as suggested by Takahashi *et al.* (1981).

Exogenously Added General Base or the Elevation of pH Did Not Restore the Dehydrogenase Activity of Mutant Enzymes. Compounds shown in other studies to function as a general base (Almarsson *et al.*, 1993) were added to the assay mixture to determine whether the dehydrogenase activity could be restored. The activities of the three mutants could not be enhanced in the presence of up to 200 mM sodium acetate, sodium formate, or imidazole.

The dehydrogenase activity as a function of pH was determined. The pH profiles of the three mutant enzymes were similar to that of native enzyme. Even at elevated pH's, where the dehydrogenase rates increased, the activities of the mutant enzymes were essentially 1000-fold lower than that of the recombinantly expressed native enzyme (Figure 4).

DISCUSSION

The accumulated data make it appear that aldehyde dehydrogenase catalyzes the oxidation of aldehydes in a manner similar to the way in which glyceraldehyde-3-phosphate dehydrogenase oxidizes its substrate. That is, a nucleophile, cysteine 302, attacks the substrate to form a hemiacetal, which is oxidized to a thioacyl intermediate (Feldman & Weiner, 1972; Weiner *et al.*, 1976; Weiner, 1979). The hydrolysis of this intermediate appears to be the rate-limiting step for many of the liver mitochondrial class 2 forms of the enzyme (Feldman & Weiner, 1972; Weiner *et al.*, 1976; Vallari & Pietruszko, 1984; Farrés *et al.*, 1994). It has been assumed, but never proven, that the hydrolysis involved general base catalysis and perhaps even a charge relay mechanism (Takahashi *et al.*, 1981), as is found with so many enzymes. Recently, we showed that the highly conserved histidine residues most likely were not the essential components of the active site (Zheng & Weiner, 1993). Studies showed that chemical modification of the conserved glutamate 268 residue inactivated the enzyme (Abriola *et al.*, 1990; Pietruszko *et al.*, 1991). This residue could be a candidate for the general base or for being part of the triad, should a charge relay mechanism be essential.

Mutation of glutamate 268 to an aspartate, glutamine, or lysine caused a dramatic decrease in the specific activity of the enzyme. Each mutant possessed less than 0.4% of the dehydrogenase activity and less than 0.8% of the esterolytic activity. Thus, not only removing the charge from position 268 but also changing the size of the negative residue greatly affected the ability of the enzyme to function properly.

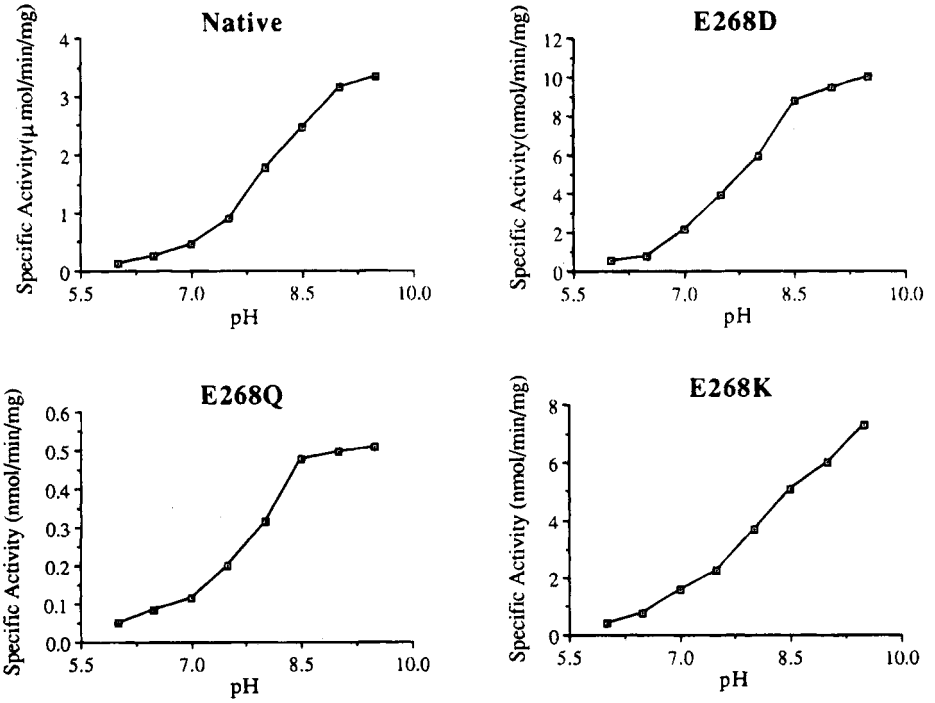


FIGURE 4: pH profiles for recombinantly expressed ALDHs. The dehydrogenase assay was performed in 100 mM sodium phosphate (pH 6.0−7.5) and 100 mM pyrophosphate (pH 8.0−9.5). The concentration of NAD was 1 mM, and the concentration of propionaldehyde was 14 μM for native and 140 μM for mutant enzymes. The units for the mutants are reported in nanomoles, while native enzyme is reported in micromoles.

The mutation at position 268 only affected the k_{cat} step and not the K_m values for either substrate. The K_m for NAD and for aldehyde were essentially unaltered in the mutants. Even the K_d for NADH was barely affected in the glutamine mutant when compared to the recombinantly expressed native enzyme. Thus, it does not appear that the residue at position 268 in human mitochondrial class 2 ALDH is interacting with the coenzyme, as we showed to occur with glutamate 487 (Farrés *et al.*, 1994).

It was suggested by Pietruszko that glutamate 268 in human liver mitochondrial aldehyde dehydrogenase was in an extremely hydrophobic environment (Abriola *et al.*, 1990; Pietruszko *et al.*, 1993). However, Loomes and Jörnvall (1991) proposed that glutamate 268 was on the surface of the enzyme. If glutamate 268 were on the surface of human liver mitochondrial class 2 ALDH or accessible to solvent, one would expect there to be a p*I* difference between the native and the mutant enzymes. If glutamate 268 were buried, changing glutamate to glutamine would not affect the p*I*. E268Q and E268K had p*I* values of 5.0, compared with 4.9 for recombinantly expressed native enzyme; this is consistent with the observation that glutamate 268 appeared to be on the surface of human liver (class 2) mitochondrial aldehyde dehydrogenase (Loomes & Jörnvall, 1991), at least in the absence of coenzyme or substrate. This finding, however, cannot be used to support a catalytic role for the residue.

If glutamate 268 were the essential general base or a component of a charge relay triad, it could function in the deacylation step or in the initial interaction of the nucleophile (cysteine 302) with the carbonyl group of the substrate. The fact that the esterolytic activity of the enzyme decreased by a factor greater than 100 in the mutants shows that the residue is not just performing an essential role in the hydride transfer step, which occurs only in the dehydrogenase reaction. For the ester reaction, we suggested that the rate-limiting step is the initial acylation, not deacylation, as it is with the dehydrogenase reaction (Weiner *et al.*, 1976). The ester hydrolysis data are consistent with a role for glutamate 268 in the initial activation of cysteine 302. Two pre-steady state kinetic assays were performed to test for this possibility. If the role of glutamate 286 were only to hydrolyze the acyl intermediate, one would expect to have found an accumulation of the acyl intermediates with the mutant enzyme. That is, there would have been essentially 1 mol of NADH or 1 mol of *p*-nitrophenol released per active site prior to the steady state portion of the reaction. Neither NADH nor *p*-nitrophenol accumulated with mutants during the first turnover, showing that the slow step was prior to, not after, the formation of the acyl intermediate. From these observations, we conclude that in human mitochondrial class 2 ALDH glutamate 268 functions in the activation of the nucleophile necessary for the initial covalent binding of enzyme and substrate.

Neither the addition of salts, which could act as a general base, nor increasing the pH of the assay medium could restore the activity of the mutant enzymes. Thus, whether the role of glutamate 268 is to be the essential general base or to be involved in the stabilization of the activated cysteine 302 thiolate ion, its presence is mandatory in order to have a highly active enzyme. Even an aspartate at position 268 could not replace the glutamate. Detailed studies performed with trypsin showed that changing either the general base (histidine 57) or aspartate 102, a component of the charge relay triad, caused a drastic decrease in the activity of the enzyme (Craik *et al.*, 1987; Corey & Craik, 1992). In the absence of the actual structure of the aldehyde dehydrogenase, it is difficult to determine the precise role of the residue. The fact that glutamate 268 is conserved in all known aldehyde dehydrogenases (Hempel *et al.*, 1993) supports the essential role of the residue in catalysis.

Over the years there has been some controversy as to whether or not the dehydrogenase and esterase reactions occurred at the same site. Our laboratory (Tu & Weiner, 1988b) and others (Deady *et al.*, 1985; Abriola & Pietruszko, 1992) have used chemical modifications to suggest that they did not. Other laboratories, for example, Loomes and Kitson (1986) and Kitson (1986), showed that the two reactions occurred at the same site. Mutational analyses of the enzyme, including serine 74 (Rout & Weiner, 1994) and cysteine 302 (Farrés *et al.*, 1995), as well as glutamate 268, all showed that there was a parallel loss of dehydrogenase and esterase activity. Thus, it appears that the two reactions catalyzed by aldehyde dehydrogenase require the same active site components and occur at the same site.

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BI941326L