Investigation of the Active Site Cysteine Residue of Rat Liver Mitochondrial Aldehyde Dehydrogenase by Site-Directed Mutagenesis†

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ABSTRACT: To determine the active site cysteine residue in aldehyde dehydrogenase, we mutated amino acid residues 49, 162, and 302 of recombinantly expressed rat liver mitochondrial (class 2) aldehyde dehydrogenase. The C49A and C162A mutants were fully active tetrameric enzymes, although the C162A mutant was found to be highly unstable. The C302A mutant was also a tetramer and bound coenzyme, but lacked both dehydrogenase and esterase activities. To test for the role of cysteine 302 as a nucleophile, the residue was mutated to a serine, a poor nucleophile. This C302S mutant was active but was a much poorer catalyst, with a k_{cat}/K_m value 7×10^5 times lower than that of the recombinant native enzyme. Unlike with native enzyme where deacylation is rate limiting, formation of the serine hemiacetal intermediate appeared to be the rate-limiting step. Cysteine 302 is the only strictly conserved cysteine residue among all the available sequences of the aldehyde dehydrogenase superfamily, supporting the role of this residue as the active site nucleophile of aldehyde dehydrogenase.

NAD(P)$^+$-dependent aldehyde dehydrogenases (ALDH)[1] (EC 1.2.1.3) catalyze the dehydrogenation of aldehydes, as well as the hydrolysis of activated esters. The aldehyde dehydrogenase superfamily includes broad substrate enzymes, such as class 1 (cytosolic), class 2 (mitochondrial), class 3 (tumor-associated), and microsomal aldehyde dehydrogenases (Dunn *et al.*, 1989; Farrés *et al.*, 1989; Hempel *et al.*, 1989; Miyauchi *et al.*, 1991), as well as substrate-specific enzymes, such as betaine aldehyde dehydrogenase and succinate, glutamate, methylmalonate or 2-hydroxymuconate semialdehyde dehydrogenases (Weretilnyk & Hanson, 1990; Boyd *et al.*, 1991; Krzywicki & Brandriss, 1984; Kedishvili *et al.*, 1992; Steele *et al.*, 1992; Nordlund & Shingler, 1990). Unlike other dehydrogenases, no three-dimensional structure of an aldehyde dehydrogenase has been elucidated, and thus the active site residues and the catalytic mechanism have not been firmly established.

Mammalian liver class 1 and class 2 aldehyde dehydrogenases were purified in the 1970s, their amino acid, cDNA and gene sequences have been reported [Farrés *et al.* (1989) and references cited therein], and thus they have been used extensively as models for kinetic and structural studies. These are tetrameric enzymes with identical subunits of molecular weight 55 000. It was originally postulated that these enzymes functioned in a manner analogous to that of glyceraldehyde-3-phosphate dehydrogenase. That is, a cysteine residue acts as a nucleophile, attacking the carbonyl group of the substrate to form a thiohemiacetal. This covalent adduct was postulated to be oxidized to a thioester when the hydride was transferred to NAD$^+$ (Weiner, 1979). Evidence to support the role of a cysteine at the active site of aldehyde dehydrogenase primarily came from spectroscopic data (Dunn & Buckley, 1985) and chemical modification studies of thiol-directed reagents (Hempel & Pietruszko, 1981).

Of all the cysteine residues that were conserved among mammalian class 1 and class 2 aldehyde dehydrogenases, three had been implicated to be at the active site by using chemical modification, followed by sequence analysis. Cys302 was labeled by iodoacetamide, and disulfiram could protect the enzyme against alkylation (Hempel *et al.*, 1982). However, complete inactivation by iodoacetamide could not be achieved (Hempel & Pietruszko, 1981), precluding definitive assignment of the active site nucleophile. Later, Cys302 was selectively modified by a coenzyme analogue, confirming its location at or close to the active site (von Bahr-Lindström *et al.*, 1985).

Our laboratory reported that substrates or competitive inhibitors could protect Cys49 and Cys162 of horse mitochondrial aldehyde dehydrogenase from modification by *N*-ethylmaleimide and suggested that these residues could be components of the active site (Tu & Weiner, 1988a). In fact, we reported that the dehydrogenase reaction seemed to be dependent upon the presence of Cys49, while a nonphysiological esterase reaction was dependent upon the involvement of Cys162 (Tu & Weiner, 1988b). Some investigators had also suggested that these two reactions occurred at separate active sites (Blackwell *et al.*, 1983), while others supported a single-site hypothesis (Kitson, 1986).

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[1] Abbreviations: C49A, C162A, C302A, C302S, and E487K, mutant aldehyde dehydrogenases where the residue at one position is replaced by another; DACA, 4-*trans*-(*N*,*N*-dimethylamino)cinnamaldehyde; rec ALDH, recombinantly expressed aldehyde dehydrogenase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide.

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Table 1: Oligonucleotides Used for Site-Directed Mutagenesis[a]

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                        49
     Gly Glu Val Ile Ala Gln Val Ala
5' G GGG GAG GTC ATC GCC CAG GTA GCC G 3'

                       162
   Pro Val Gly Val Ala Gly Gln Ile Ile
5' CCT GTG GGC GTG GCC GGA CAG ATC ATT 3'
                  ^
                EaeI

                       302
                       Ala
     Gln Gly Gln Cys Ser Cys Ala Gly Ser
                       G
5' C CAG GGC CAG TGC TCA TGT GCG GGC TCC C 3'
                    ^
                  FspI
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[a] Amino acid sequences of rat class 2 aldehyde dehydrogenase (Farrés *et al.*, 1989) around the amino acid changed are shown above the corresponding oligonucleotide sequences. Mutated nucleotides are underlined. New restriction sites created as a result of mutagenesis are indicated (). A 2-fold-degenerated oligonucleotide was used to produce both the C302A and C302S mutants in a single mutagenesis experiment.

Substrate-based affinity reagents, such as bromoacetophenone (Abriola *et al.*, 1990) and a vinyl ketone analogue (Blatter *et al.*, 1990), also located Cys302 to be near or at the active site. More recently, Cys302 was labeled by 4-nitrophenyl dimethylcarbamate (Kitson *et al.*, 1991) and by two substrates, 4-*trans*-(*N,N*-dimethylamino)cinnamaldehyde (DACA) and 4-*trans*-(*N,N*-dimethylamino)cinnamoylimidazole; it was concluded that dehydrogenase and esterase activities occur at a single site (Blatter *et al.*, 1992). Other residues have been labeled, however, with these reagents. For example, Glu268 was initially identified as being essential because it reacted with bromoacetophenone (Abriola *et al.*, 1987), and Ser74 reacted with DACA (Loomes *et al.*, 1990).

Site-directed mutagenesis has become a powerful alternative technique to investigate the role of an individual residue on an enzyme. We presented the cDNA sequence (Farrés *et al.*, 1989) and the expression of class 2 aldehyde dehydrogenase in *Escherichia coli* (Jeng & Weiner, 1991). Site-directed mutagenesis was performed to assign a role for Glu487 in coenzyme binding (Farrés *et al.*, 1994) and to investigate the roles of the conserved histidines (Zheng & Weiner, 1993), serine 74 (Rout & Weiner, 1994), and glutamate 268 (Wang & Weiner, 1995). Here, we report the results of a study where three cysteine residues of rat liver mitochondrial aldehyde dehydrogenase were converted to alanine residues. The residue that caused the enzyme to become inactive was also mutated to a serine residue in order to study the kinetic effects of the presence of a different nucleophile at the active site.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Oligonucleotide-directed mutagenesis was performed by the method of Kunkel *et al.* (1987), following instructions supplied with the MutaGene kit (Bio-Rad). The oligonucleotides used are presented in Table 1. Screening for the desired mutations (underlined) was carried out by colony hybridization using the mutagenic oligonucleotides as probes. When possible, new restriction sites were introduced and used to test for the presence of the mutations. Mutations were verified by M13 DNA sequencing (Sanger *et al.*, 1977), using synthetic oligonucleotides as primers. DNA fragments containing the desired mutations were excised from M13mp19, gel-purified, and exchanged for those of native rat liver mitochondrial aldehyde dehydrogenase cDNA in the expression plasmid for the mature enzyme (Jeng & Weiner, 1991).

Expression in E. coli and Purification of Recombinant Aldehyde Dehydrogenases. Previously, we reported the construction of plasmids, expression in *E. coli,* and purification of recombinant mature rat aldehyde dehydrogenase (Jeng & Weiner, 1991). All the mutant proteins were purified following the same procedure as that used for the recombinant native enzyme. Purified proteins were kept at 4 or −20 °C in the presence of 50% glycerol and 0.025% 2-mercaptoethanol. An HPLC TSK-G3000SW column (Tosoh-Haas), equilibrated with 150 mM sodium phosphate (pH 7.0), was used to remove Ampholites and 2-mercaptoethanol from the purified proteins prior to their use.

Enzyme Assay and Protein Determination. Aldehyde dehydrogenase activity was determined at 25 °C in 50 mM sodium pyrophosphate (pH 9.0) or 50 mM sodium phosphate (pH 7.4). Propionaldehyde was used as a substrate for the dehydrogenase activity, and reduction of NAD^+ was monitored at 340 nm. Esterase activity was measured at 400 nm, using 50 mM sodium phosphate (pH 7.4) as a buffer and 125 μM 4-nitrophenyl acetate as a substrate. The extinction coefficient of 9.45×10^3 M^{-1} cm^{-1} was used for 4-nitrophenol. Inactive mutant proteins were detected by dot blotting and Western blotting techniques, using anti-rat aldehyde dehydrogenase antiserum as previously described (Farrés *et al.*, 1989). To quantitate the enzyme amount in dot blots, nitrocellulose was subjected to densitometer (Ultroscan, LKB) scanning.

Activation by magnesium ions was tested by adding 2.5 mM magnesium chloride at pH 7.4, prior to the addition of substrate. Inhibition by chloral hydrate was determined by adding the inhibitor to a final concentration of 10 mM. Protein concentration was determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard. Concentration of aldehyde dehydrogenase was also measured from its absorption spectrum or from the area under the elution peak after an analytical HPLC TSK-G3000SW column, using $\epsilon = 2.25 \times 10^5$ M^{-1} cm^{-1} (Greenfield & Pietruszko, 1977).

Kinetic Measurements. Kinetic constants were determined by fitting the initial rates to the Michaelis−Menten equation using least-squares analysis. Dixon plots were used to determine K_i values for chloral hydrate. Pre-steady state burst was determined at pH 7.4 as described (Weiner *et al.*, 1976), using an Aminco filter fluorometer.

Pulse−Chase Labeling of E. coli-Expressed Aldehyde Dehydrogenase. Labeling of recombinant native and mutant aldehyde dehydrogenases was performed essentially as previously described (Jeng & Weiner, 1991). After incubation with [^{35}S]methionine (New England Nuclear), cells were washed and incubated at 37 °C in medium containing 1 mg/mL cold methionine. Aliquots (10 μL) were removed at various times, chilled on ice, and lysed in an equal volume of Laemmli buffer (Laemmli, 1970). Samples were subjected to SDS−PAGE and autoradiography. The amount of protein was quantitated by densitometer (Response II, Gilford) scanning.

Molecular Weight Determination. The molecular weights of recombinant rat aldehyde dehydrogenase and of the mutant

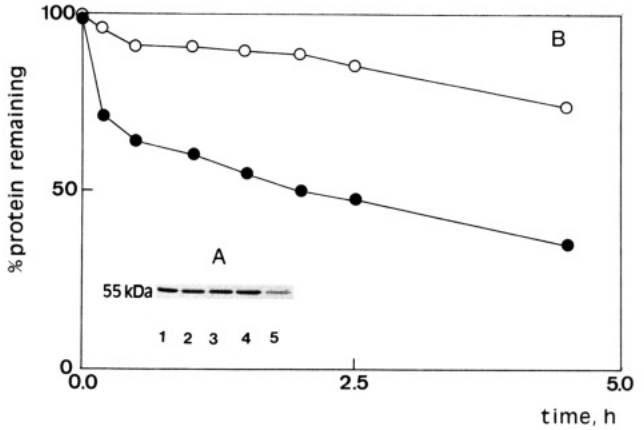


FIGURE 1: (A) Analysis of *E. coli*-expressed aldehyde dehydrogenase by SDS−PAGE and Western blot analysis. Lanes: 1, recombinant native aldehyde dehydrogenase; 2, C49A mutant; 3, C302A mutant; 4, C302S mutant; 5, C162A mutant. Samples were separated on a 10% gel and transferred to a nitrocellulose membrane. Aldehyde dehydrogenase was detected using anti-rat mitochondrial aldehyde dehydrogenase antiserum. Marker proteins (not shown) were used to calibrate the gel. The subunit molecular weight was found to be 55 000. (B) Determination of stability of the recombinant native aldehyde dehydrogenase (○) and the C162A mutant (●) by pulse−chase labeling.

forms were estimated by gel filtration chromatography on a Sephacryl S-300 Superfine column (1.8 × 90 cm) or by an HPLC TSK-G3000SW column and 5−25% gradient polyacrylamide gel electrophoresis.

Agarose Gel Isoelectric Focusing. Analytical isoelectric focusing was performed as previously described (Guan *et al.*, 1988).

NADH Binding. Binding of NADH to aldehyde dehydrogenase was followed in an Aminco SPF-500 spectrofluorometer, measuring the fluorescence emission at 460 nm with excitation at 340 nm. A control titration without enzyme was carried out for the blank determination. Concentrations of NADH added were corrected for dilution. The concentration of the stock solution of NADH was determined spectrophotometrically at 340 nm using an extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}. Data were treated as described (Takahashi *et al.*, 1980) for the calculation of K_d for NADH.

RESULTS

Expression of Aldehyde Dehydrogenase Mutants in E. coli. It was previously reported that rat liver mitochondrial aldehyde dehydrogenase could be expressed in *E. coli* as an active tetrameric enzyme when the cDNA was cloned into the pT7-7 expression plasmid, under the control of the T7 RNA polymerase promoter (Jeng & Weiner, 1991). The recombinantly expressed enzyme had essentially the same physicochemical and kinetic properties as the enzyme isolated from rat liver (Jeng & Weiner, 1991; Farrés *et al.*, 1994; Rout & Weiner, 1994; Zheng & Weiner, 1993).

The three alanine-containing mutants, C49A, C162A, and C302A, were expressed successfully using the same procedure, as determined by SDS−PAGE followed by Western blot analysis (Figure 1A). It could be estimated that the activity from the homogenate containing the C49A mutant was similar to that containing the recombinant native enzyme. Approximately 10% of this activity was found in the homogenate corresponding to the C162A mutant. No catalytic activity was detected in the homogenate from the cells expressing the C302 mutant.

Western blot analysis of samples separated by SDS−PAGE revealed that similar amounts of immunoreactive protein were obtained when the C49A and C302A mutants were compared to the amount of expressed recombinant native enzyme. In contrast, the yield of immunoreactive protein was only *ca.* 10% when the C162A mutant was compared to others (Figure 1A), consistent with the lower level of activity found in homogenates. Pulse−chase analysis revealed that the C162A mutant was less stable in *E. coli* than was the native enzyme (Figure 1B), thus explaining the lower yield.

The crude *E. coli* homogenates were subjected to isoelectric focusing and assayed for the presence of both immunoreactive and catalytically active protein. All three mutants tested positive by Western blotting, consistent with what was observed after SDS−PAGE. It was not possible to detect any activity band for the C302A mutant, but both the recombinant native enzyme and the C49A mutant were catalytically active. Very little protein corresponding to the C162A mutant was present (data not shown).

Physicochemical Properties of the Aldehyde Dehydrogenase Mutants. The subunit molecular weights of the recombinant native enzyme and the mutant proteins, as determined by SDS−PAGE (Figure 1A), were 55 000, consistent with their amino acid sequence (Farrés *et al.*, 1989). Gradient PAGE showed that both the recombinant native enzyme and the mutant proteins had molecular weights of 220 000, indicating that all were in the tetrameric form. The same result was obtained by using gel filtration chromatography.

Purification of the Expressed Mutant Forms of Aldehyde Dehydrogenase. It was necessary to use dot blots to follow the course of purification of the inactive C302A mutant or the unstable C162A mutant, whereas it was possible to use dehydrogenase activity for the identification of the fully active C49A form. The recombinant native enzyme and the three mutant proteins could be eluted from a DEAE-cellulose column by the same salt gradient. The mutant proteins bound as well to a Cibacron Blue−Sepharose affinity column as did the recombinant native enzyme, suggesting that the affinity for NAD^+ was not altered as a result of the mutations. The proteins eluting from the affinity column still were not pure as judged by SDS−PAGE. It was necessary to employ preparative isoelectric focusing to obtain homogeneous mitochondrial enzymes, as oringinally used to purify the horse liver mitochondrial enzyme (Feldman & Weiner, 1972). All three mutants had the same p*I* (5.2 ± 0.1) as that of the recombinant native enzyme.

Kinetic Properties of the Active Mutant Forms of Aldehyde Dehydrogenase. No dehydrogenase or esterase activity could be detected using highly purified preparations of the C302A mutant (Tables 2 and 3). The lower level of detection would have been 0.01%. The active mutants, C49A and C162A, were found to have k_{cat} values essentially equal to that of the recombinant native enzyme. Even the K_m values for propionaldehyde and NAD^+ were unaltered (Table 2). The k_{cat} value (46 min^{-1}) for the esterase reaction catalyzed by the enzyme was not affected by either of these mutations. Other aspects of the overall reaction remained unchanged in the active mutant forms. These included the activation by Mg^{2+} ions and the magnitude of the pre-steady state burst (Table 3), which have been shown to occur for the recombinant native enzyme (Farrés *et al.*, 1994). Thus, in spite of the chemical modification data (Tu & Weiner,

Table 2: Kinetic Constants for *E. coli*-Expressed Aldehyde Dehydrogenase at pH 9.0

	K_m (μM)		k_{cat}	k_{cat}/K_m[c]
	propionaldehyde[a]	NAD[b]	(min^{-1})	(μM^{-1} min^{-1})
rec ALDH	0.15	78	110	730
C49A	0.15	93	101	670
C162A	*d*	*d*	100	*d*
C302A	*e*	*e*	*e*	*e*
C302S	800	12	0.8	0.001

[a] NAD^+ concentration was 0.8 mM. [b] Propionaldehyde concentration was 100 μM, except for the C302S mutant where 12 mM propionaldehyde was used. [c] K_m for aldehyde. [d] Not determined, due to the low amount of protein available after purification. The value for k_{cat} was estimated using saturating concentrations of propionaldehyde (100 μM) and NAD^+ (0.8 mM). [e] No dehydrogenase activity could be detected using a concentration as high as 0.5 μM C302A mutant in the assay.

Table 3: Properties of *E. coli*-Expressed Aldehyde Dehydrogenase

	NADH burst	Mg^{2+} activation	esterase activity
rec ALDH	+	+	+
C49A	+	+	+
C162A	*a*	+	+
C302A	ND[b]	ND	ND[c]
C302S	ND	ND	ND[c]

[a] Not determined. [b] ND, not detectable. [c] No esterase activity could be detected, even using as much as 0.12 μM C302A mutant or 0.8 μM C302S mutant in an assay.

1988a,b), it appears that these two residues, Cys49 and Cys162, are not involved in the catalytic process, but the absence of Cys162 seems to affect the stability of the enzyme.

Coenzyme Binding to the Inactive C302A Mutant. During the purification procedure it was found that the C302A mutant bound to a Cibacron Blue−Sepharose affinity column and was eluted with NAD^+. This suggests that the coenzyme binding site might not be affected by the presence of the alanine residue, despite the potential proximity of residue 302 to the nicotinamide ring of coenzyme (von Bahr-Lindström *et al.*, 1985). A fluorometric binding assay was used to further test for binding of coenzyme to the expressed rat enzyme. It had been demonstrated that the fluorescence of NADH bound to horse liver mitochondrial ALDH was increased over that of the free coenzyme (Takahashi *et al.*, 1980). A similar increase was found with the recombinant native rat liver enzyme (Rout & Weiner, 1994). The inactive C302A mutant also bound NADH (2.3 mol of NADH/mol of tetrameric enzyme) and the K_d was 18 μM, compared to 2.2 mol of NADH/mol and $K_d = 2$ μM for the native enzyme.

Expression, Purification, and Catalytic Properties of the C302S Mutant. The accumulated data made it appear that the essential cysteine residue was located at position 302. It has been postulated that the role of this residue is to function as a nucleophile (Weiner, 1979; Hempel & Pietruszko, 1981). To test for a catalytic role for a nucleophile at this position, Cys302 was converted to a serine residue. The methods used to construct, express, and purify this mutant were identical to those used for the C302A mutant. Little, if any, catalytic activity was detected in the crude homogenate. The purified enzyme, however, was found to be an active enzyme, but k_{cat} was just 1% that of native enzyme. The K_m for aldehyde increased dramatically while the K_m for NAD^+ decreased slightly (Table 2). No esterase activity could be detected with the C302S mutant (Table 3). Chloral hydrate, a competitive inhibitor, postulated to form a covalent complex with the enzyme (Weiner, 1979; Feldman & Weiner, 1972), was a poorer inhibitor of the C302S mutant ($K_i = 17.5$ mM) than it was of the native enzyme ($K_i = 0.7$ μM).

Unlike the recombinant native enzyme (Farrés *et al.*, 1994), the activity of the C302S mutant was not affected by the presence of Mg^{2+} ions, nor was there a pre-steady state burst of NADH formation (Table 3). These observations imply that the rate-limiting step for the enzyme may have changed when the cysteine was converted to a serine residue. The rate-limiting step for the mutant does not appear to be hydride transfer, as chloroacetaldehyde was oxidized 12-fold more rapidly than was propionaldehyde. As argued previously (Farrés *et al.*, 1994; Feldman & Weiner, 1972), it is more difficult to oxidize the chloro derivative, so that if hydride transfer were rate limiting, propionaldehyde would have been oxidized more rapidly than chloroacetaldehyde.

DISCUSSION

The oxidation of an aldehyde to an acid usually proceeds through a covalent intermediate (Weiner, 1979). With formaldehyde dehydrogenase, the adduct is a thiohemiacetal formed between the substrate and glutathione (Koivusalo *et al.*, 1989). With glyceraldehyde-3-phosphate dehydrogenase, the adduct is a thiohemiacetal formed between the substrate and a cysteine residue (Harris & Waters, 1976). All investigators studying the oxidation of aldehydes by NAD(P)$^+$-dependent aldehyde dehydrogenases have concluded that the reaction also proceeds through covalent catalysis, and it has been assumed that the active site nucleophile is a cysteine residue (Weiner, 1979). There have been various reports as to which of the cysteine residues of aldehyde dehydrogenase is actually the component of the active site (Hempel *et al.*, 1982; von Bahr-Lindström *et al.*, 1985; Tu & Weiner, 1988a,b). Recently, Cys302 has been labeled by trapping the acyl-enzyme intermediates formed with both aldehyde and ester substrates (Kitson *et al.*, 1991; Blatter *et al.*, 1992). In contrast, protection studies allowed Cys49 and Cys162 to be identified (Tu & Weiner, 1988a,b).

The data obtained from mutants prepared by site-directed mutagenesis presented in this study support the conclusion made by others that Cys302 is a component of the active site. Conversion of either Cys49 or Cys162 to alanine did not cause a loss of catalytic activity to occur. Conversion of Cys302 to an alanine residue caused a total loss of both the dehydrogenase and esterase activities. Apparently, none of the adjacent cysteine residues in class 2 aldehyde dehydrogenases, Cys301 or Cys303 (Table 4), could substitute for Cys302 as a nucleophile, despite the fact that Cys301 had shown some reactivity toward alkylating thiol reagents (Hempel *et al.*, 1982; Johansson *et al.*, 1992). This emphasizes that Cys302 is uniquely positioned for catalysis at the active site of aldehyde dehydrogenase.

It is not obvious why our previous chemical modification study (Tu & Weiner, 1988a,b) led to an incorrect conclusion about the role of cysteine at position 49 or 162. The finding that the C162A mutant was unstable suggests that at least this residue might be important in folding or maintaining the proper conformation of the enzyme. It is possible that the introduction of the large, relatively hydrophobic *N*-ethylsuccinimidyl group of NEM, not the loss of the SH group, was the cause of the inactivation that we observed. Experiments in progress indeed indicate that substitution of these cysteines by tryptophan rather than alanine residues

Table 4: Comparison of Amino Acid Sequences of the Aldehyde Dehydrogenase Superfamily Aligned at Position 302 of Rat Class 2 Aldehyde Dehydrogenase[a]

Rat class 2 ALDH	295	FFNQGQCCCAGSRTFVQE
Human ALDHx	295	FFNMGQCCCAGSRTFVEE
A. nidulans AldA	291	FFNHGQCCCAGSRILVQE
Rat class 1 ALDH	296	FFHQGQICVAASRLFVEE
Rat 10-FTHFD	700	FFNKGENCIAAGRLFVEE
E. coli ALDH	295	FYNQGQVCIAGTRLLLEE
V. cholerae ALDH	294	FFNQGEVCTCPSRILVHE
Spinach BADH	284	FWTNGQICSATSRLLVHE
E. coli BADH	280	FFSSGQVCTNGTRVFVPA
Yeast P5CDH	344	FEFQGQKCSAASRLYLPE
E. coli SSDH	282	FRNAGQTCVCANRLYVQD
P. oleovorans AlkH	253	FSNNGQTCIAPDHVFVHR
A. eutrophus AcDHII	294	ALNQGEVCTCPSRALIQE
E. coli Ald	278	VINSGQVCNCAERVYVQK
P. putida HMSD	281	FANGGQVCLGTERLYVER
Rat MMSD	278	FGAAGQRCMALSTAVLVG
Rat tumor-specific ALDH	236	FMNSGQTCVAPDYILCDP
E. histolytica ALDH	300	SINAGQICVCVDHVFVPR
Rat microsomal ALDH	234	YMNCGQTCIAPDYILCEA
P. aeruginosa MMSD	274	VGAAGQRCMAISVAVLVG
Pea protein 26G	293	VGTAGQRCTTCRRLYLHE

[a] Conserved residues between rat class 2 aldehyde dehydrogenase and other enzymes are highlighted. The strictly conserved cysteine is shaded. From top to bottom: rat mitochondrial ALDH (Farrés *et al.*, 1989), human ALDHx (Hsu & Chang, 1991), *Aspergillus nidulans* ALDH (Pickett *et al.*, 1987), rat phenobarbital-inducible ALDH (Dunn *et al.*, 1989), rat 10-formyltetrahydrofolate dehydrogenase (Cook *et al.*, 1991), *E. coli* putative ALDH (Heim & Strehler, 1991), *Vibrio cholerae* ALDH (Parsot & Mekalanos, 1991), spinach betaine aldehyde dehydrogenase (Weretilnyk & Hanson, 1990), *E. coli* betaine aldehyde dehydrogenase (Boyd *et al.*, 1991), *Saccharomyces cerevisiae* pyrroline-5-carboxylate dehydrogenase (Krzywicki & Brandriss, 1984), *E. coli* succinate semialdehyde dehydrogenase (submitted by E. Niegemann, A. Schulz, and K. Bartsch to EMBL/GenBank data banks with the accession number M88334), *Pseudomonas oleovorans* AlkH (Kok *et al.*, 1989), *Alcaligenes eutrophus* acetaldehyde dehydrogenase II (Priefert *et al.*, 1991), *E. coli* lactaldehyde dehydrogenase (Hidalgo *et al.*, 1991), *Pseudomonas putida* 2-hydroxymuconic semialdehyde dehydrogenase (Nordlund & Shingler, 1990), rat methyl malonate semialdehyde dehydrogenase (Kedishvili *et al.*, 1992), rat tumor-specific ALDH (Hempel *et al.*, 1989), *Entamoeba histolytica* ALDH (submitted by W. W. Zhang and J. Samuelson with the accession number L05667), rat microsomal ALDH (Miyauchi *et al.*, 1991), *Pseudomonas aeruginosa* methyl malonate semialdehyde dehydrogenase (Steele *et al.*, 1992), and pea turgor-responsive protein 26G (Guerrero *et al.*, 1990).

produces inactive enzymes (Ghenbot & Weiner, 1991). In this regard, it also has been found by others that the replacement of cysteine by site-directed mutagenesis can lead to conclusions different from those reached by chemical modification studies, especially when a bulky thiol reagent such as NEM is used (Profy & Schimmel, 1986; Kato *et al.*, 1988; Jordan-Starck & Rodwell, 1989; Knowles *et al.*, 1992).

A dehydrogenase could be catalytically inactive for a variety of reasons, including those associated with conformational changes. If NAD^+ could no longer bind or NADH not dissociate, an inactive enzyme would be formed. If the monomers could not assemble into tetramers, an inactive protein would exist (Jeng & Weiner, 1991). None of these were the cause of the C302A mutant being inactive. The enzyme was a tetramer that was stable to purification. More important were the observations that the C302A mutant bound to a Cibacron Blue−Sepharose affinity column and was eluted with NAD^+ and that NADH could bind to the enzyme as determined by a fluorimetric binding assay. Thus, it appears that the reason for the inactivity of the enzyme was that Cys302 functions at the active site, presumably as the essential nucleophile residue.

There has been much debate in the literature as to whether or not the dehydrogenase and the esterase reactions occur at the same site (Tu & Weiner, 1988b; Blackwell *et al.*, 1983; Kitson, 1986; Kitson *et al.*, 1991; Blatter *et al.*, 1992; Duncan, 1985). Our previous chemical modification studies lead us to suggest that different nucleophilic residues were involved in the two reactions. Opposite conclusions now must be made on the basis of the fact that removal of one single cysteine group caused the loss of both activities.

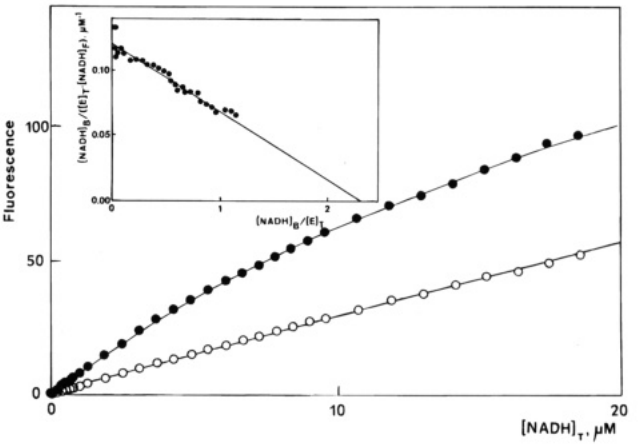


FIGURE 2: Fluorescence enhancement titration curves of NADH binding to the C302A mutant. The titration was carried out in 100 mM sodium phosphate (pH 7.4) at 25 °C. Protein concentrations were between 0.88 and 0.95 μM. The solution was excited at 340 nm, and the emission intensity was recorded at 460 nm. (●) NADH fluorescence in the absence of protein; (○) NADH fluorescence of the binary complex. Inset: Scatchard plot corresponding to the same data. $[E]_T$, concentration of C302A mutant; $[NADH]_T$, $[NADH]_B$, and $[NADH]_F$ are concentrations of total, bound, and free NADH, respectively. The constants obtained were, for recombinant native enzyme, $K_d = 2$ μM and $n = 2.1$ (Rout & Weiner, 1994) and, for C302A, $K_d = 18$ μM and $n = 2.3$.

Recently, the active site cysteine of glyceraldehyde-3-phosphate dehydrogenase was also mutated to an alanine residue (Corbier *et al.*, 1992). It was reported that the mutant retained some dehydrogenase activity, although it completely lost the esterase activity. The authors suggested that the enzyme could function through an alternative catalytic mechanism in which the true substrate was the *gem*-diol form of the aldehyde. Even though propionaldehyde forms a *gem*-diol, this mechanism apparently does not exist in aldehyde dehydrogenases, since the C302A mutant lacked both dehydrogenase and esterase activities.

To gain further insight into the role of the nucleophilic residue at position 302, the cysteine was mutated to a serine. A serine substituted at position 302 appears to be able to function as the active site nucleophile. This was not unexpected, since a hydroxyl group can form a hemiacetal with an aldehyde, and many proteases and esterases have a serine residue at their active sites rather than a cysteine (Brenner, 1988; Gorbalenya *et al.*, 1989). Other examples of exchanging cysteine for serine at the active site of enzymes include those of thiolase (Thompson *et al.*, 1989), dienelactone hydrolase (Pathak *et al.*, 1991), and aspartate-β-semialdehyde dehydrogenase (Karsten & Viola, 1992). In each case, the mutated enzyme was active but was a poor catalyst compared to the native enzyme. Indeed, the C302S mutant was also a much poorer catalyst. The K_m for aldehyde and the K_i for the competitive inhibitor chloral hydrate increased dramatically. Overall, k_{cat}/K_m decreased 7×10^5 times with respect to the recombinant native enzyme.

The rate-limiting step for rat liver mitochondrial aldehyde dehydrogenase has not been determined with certainty. The fact that a pre-steady state burst of NADH was found showed that the rate-limiting step occurred after NADH was formed

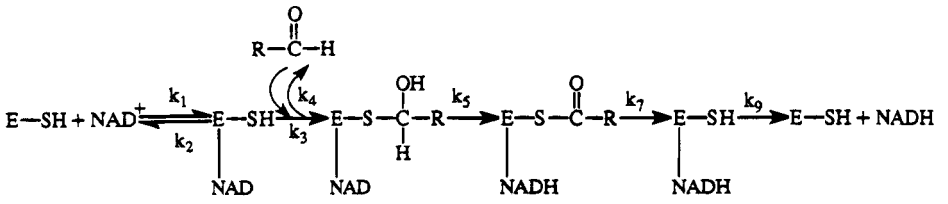
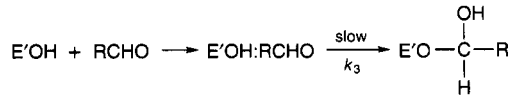


FIGURE 3: Kinetic model for the reaction catalyzed by aldehyde dehydrogenase. In the scheme is presented a model showing the native (cysteine 302) enzyme where it is postulated that the aldehyde forms a thiohemiacetal with the enzyme. For the C302S mutant, a similar model would exist but the nucleophile would be a hydroxyl group.

(Farrés *et al.*, 1994). For the horse liver mitochondrial enzyme, it was proposed that the rate-determining step was deacylation (Weiner *et al.*, 1976). Among the data supporting deacylation as the rate-limiting step rather than dissociation of NADH, as was proposed for the human liver cytosolic enzyme (Vallari & Pietruszko, 1981), is the fact that the k_{cat} was a function of the nature of the substrate (Feldman & Weiner, 1972). Aldehydes with electron-withdrawing groups were oxidized more rapidly than those without. It was shown that the rat liver enzyme oxidized chloroacetaldehyde more rapidly than acetaldehyde, and a pre-steady state burst of NADH exists (Farrés *et al.*, 1994), supporting the idea that deacylation could be the rate-limiting step for the rat liver enzyme.

No pre-steady state burst of NADH was found with the C302S mutant, suggesting that the rate-limiting step occurred prior to substrate oxidation and NADH formation. Furthermore, chloroacetaldehyde was oxidized more rapidly than was propionaldehyde. A good candidate for the rate-limiting step would be the formation of the hemiacetal intermediate when a less nucleophilic residue (serine) was at the active site.



A model to explain the kinetic behavior of the recombinantly expressed native enzyme has been presented (Farrés *et al.*, 1994) and is repeated in Figure 3. The reaction catalyzed by aldehyde dehydrogenase is irreversible, and thus k_6 can be omitted. By using the King−Altman approach (Plowman, 1972), for an ordered bi−bi reaction it can be deduced that

$$k_{cat} = k_5k_7k_9/(k_7k_9 + k_5k_9 + k_9k_7)$$

$$K_{m(aldehyde)} = k_7k_9(k_4 + k_5)/k_3(k_7k_9 + k_5k_9 + k_5k_7)$$

$$k_{cat}/K_{m(aldehyde)} = k_3k_5/(k_4 + k_5)$$

If one assumes with the native enzyme, where aldehyde binds tightly, that k_5 is greater than k_4, then the $k_{cat}/K_{m(aldehyde)} = k_3$. It can be estimated that the value for k_3 is *ca.* 700 μM^{-1} min^{-1}. In the serine mutant the rate constant for the formation of the covalent hemiacetal intermediate is given by k_{cat}, which is just 0.8 min^{-1}. Thus, changing the cysteine 302 to serine causes nearly a 1000-fold decrease in the ability of the mutant enzyme to form the covalent intermediate. The finding that the C302S mutant could not hydrolyze nitrophenyl acetate is consistent with the serine being a much poorer nucleophile, and the enzyme just could not be acylated by the ester substrate at an appreciable rate.

We recently demonstrated that changing glutamate 487 in the enzyme to a lysine, as is found in many Asian persons, resulted in a large increase in K_m for NAD^+ (Farrés *et al.*, 1994). We further showed that the altered binding of coenzyme decreased the nucleophilicity of the active site cysteine by a factor of *ca.* 20. In this study, we show that the K_m value for NAD^+ was not increased in the C302S mutant. However, the rate constant for binding of NAD^+ to the enzyme (k_1), as calculated from $k_{cat}/K_{m(NAD)}$, decreased 20-fold in the mutant. Thus, changing cysteine to serine not only decreased the k_{cat} value, reflecting the poorer nucleophilicity of the active site, but affected the rate of binding of the coenzyme to the enzyme.

The number of amino acid sequences of aldehyde dehydrogenases and evolutionary related enzymes has been consistently increasing during the recent years (Hempel *et al.* 1993a,b). Sequence comparison often provides information about essential amino acid residues. Cys302 is the only cysteine residue strictly conserved among all the sequences of the aldehyde dehydrogenase superfamily (Table 4). The only exception to this rule is the recently reported pyrroline-5-carboxylate dehydrogenase domain of the PutA protein from *Salmonella typhimurium* (Allen *et al.*, 1993). Careful analysis of the nucleotide sequence, however, reveals that, by changing the reading frame around nucleotide 2725, the sequence could be much better aligned with those listed in the table and that the equivalent to Cys302 is also present.

Recently, it was reported that the 302 region of aldehyde dehydrogenases could be aligned to the active site cysteine of thiol proteases (Hempel *et al.*, 1991). The fact that both types of enzymes have thiolesterase activity lends further support to the notion that Cys302 is also the site of ester hydrolysis. In this regard, aldehyde dehydrogenases do not have any strictly conserved histidine residue, such as those found in thiol/serine proteases, that could act as a general acid−base catalyst. Mutation analysis showed that the partially conserved His29 and His235 were not involved in catalysis (Zheng & Weiner, 1993). Glu268, another residue identified by chemical modification (Abriola *et al.*, 1987), is also largely conserved in aldehyde dehydrogenases, with the exception of methyl malonate semialdehyde dehydrogenases (Kedishvili *et al.*, 1992; Steele *et al.*, 1992). Replacement of the residue in aldehyde dehydrogenase with an alanine or lysine caused a 1000-fold loss of enzymatic activity; it will be suggested that this residue could function as an essential general base needed to ionize cysteine 302 (Wang & Weiner, 1995).

The data presented in this study lead us to suggest that cysteine 302 is indeed the active site nucleophile. Recently however, evidence was presented to suggest that serine 74 is the essential nucleophilic residue (Loomes *et al.*, 1990). This residue was modified by DACA, a chromophoric substrate, but is not very well conserved among nonmam-

malian aldehyde dehydrogenases. Mutation of the serine residue to an alanine produced an active enzyme (Rout & Weiner, 1994), showing that this residue is not essential. Thus, among all the candidates, only Cys302 can be considered to be the active site nucleophile, functioning for both the dehydrogenase and esterase reactions of aldehyde dehydrogenase. The conclusions reached in this study with class 2 aldehyde dehydrogenase should also be applicable to other broad substrate and substrate-specific aldehyde dehydrogenases. In this regard, residues flanking Cys302 appear to be highly variable (Table 4) and could have a role in defining the substrate specificities of various enzymes. Future investigation of selected conserved and variable residues should provide further insight into the detailed catalytic mechanism and substrate specificity of aldehyde dehydrogenases.

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