Involvement of Serine 74 in the Enzyme–Coenzyme Interaction of Rat Liver Mitochondrial Aldehyde Dehydrogenase†

Ujjwal K. Rout and Henry Weiner*

Biochemistry Department, Purdue University, West Lafayette, Indiana 47907-1153

Received January 21, 1994; Revised Manuscript Received May 10, 1994®

ABSTRACT: It has been suggested that the active site nucleophile in sheep liver aldehyde dehydrogenase was not a cysteine residue but was a serine located at position 74 [Loomes, K. M., Midwinter, G. G., Blackwell, L. F., & Buckley, P. D. (1990) *Biochemistry 29*, 2070–2080]. This enzyme form has not yet been cloned and expressed, but since the rat liver mitochondrial enzyme has been and shares 70% sequence homology with other cytosolic aldehyde dehydrogenases, the residue in the rat enzyme was converted into an alanine to test for the necessity of a hydroxyl group at that position. The recombinantly expressed mutant enzyme possessed 10% catalytic activity, but the K_m for NAD increased from 10 to 1900 μM while the K_ms for various aldehydes were unchanged. Kinetic analysis revealed that the dissociation constant for NAD also increased in the mutant as did k_1, the *on* velocity for NAD binding. The mutant enzyme bound poorly to an AMP–Sepharose column and did not interact as well with NADH, as determined by fluorescence enhancement binding studies, or with ADP-ribose, a competitive inhibitor. Pulse–chase analysis showed that the mutant was as stable as was the recombinantly expressed native enzyme. It was less stable to heat denaturation at 50 °C (half-life of 1 min compared to 4). Converting the alanine to a cysteine or a threonine did not restore native-like properties of the enzyme. These mutants had kinetic properties very similar to those of the alanine mutant. The three mutants had very little esterase activity compared to the recombinantly expressed native enzyme (2–5%), but the activity could be stimulated by the addition of coenzyme. The stimulated activity was between 8% and 25% of that obtained with the expressed native enzyme. The fact that the mutants possessed catalytic activity shows that serine 74 is not the essential nucleophile in the active site of the enzyme. It appears that the alteration produced by removing serine 74 affects the coenzyme binding, but this alteration also affects the active site nucleophile even in the absence of coenzyme since the mutant enzyme has a lowered specific esterase activity.

Over the past decade various research groups have been trying to elucidate the mechanism of aldehyde dehydrogenase (ALDH;[1] EC 1.2.1.3) and to determine the components of the active site. After intially isolating the enzyme, this laboratory suggested that the mechanism of action of ALDH was similar to that of glyceraldehyde-3-phosphate dehydrogenase (Feldman & Weiner, 1972). That is, a cysteine residue reacted with the aldehyde to form a hemiacetal which was oxidized to an enzyme-bound acyl intermediate. There was some disagreement in the literature as to which cysteine residue was the essential one. At the time of doing the chemical modification work the sequence of only two mammalian enzymes were known based upon the work from Jörnvall's laboratory (Hempel *et al.*, 1985). With the advent of molecular biological techniques, a large number of sequences of NAD(P)-dependent aldehyde dehydrogenases have been published (Hempel *et al.*, 1993). It now is apparent that the suggestion made by our laboratory that cysteines 49 and 162 were essential (Tu & Weiner, 1988) cannot be correct for those residues are now known to not be conserved among all the enzyme forms. Pietruszko's laboratory presented data which showed that cysteine 302 could be the essential one (Blatter *et al.*, 1990). This is the only conserved cysteine among all the known ALDHs. We substituted an alanine for the cysteine and indeed found that the mutant enzyme was void of activity (Weiner et al., 1991), thus supporting their claim.

A chromophoric substrate, DACA, reportedly labeled sheep liver cytosolic enzyme at serine 74 (Loomes *et al.*, 1990). Other investigators reported that, using a similar reagent, cysteine 302 was attacked (Abriola *et al.*, 1990). To investigate the possible role of serine 74, we converted the residue in rat liver mitochondrial ALDH to an alanine, among others, and investigated the properties of the mutant enzymes. Since mammalian ALDHs share high sequence identity, the data we present here should be relevant as to what might be found with the sheep liver enzyme.

MATERIALS AND METHODS

Reagents. Enzymes used in the molecular biology experiments were purchased from New England Biolab, Promega, Boeringer-Mannheim, or BRL. Sequencing reactions were performed using Sequanase version II from USB. Prestained molecular weight standards were from Bio-Rad. Isoelectric focusing materials (IEF–agarose and Pharmalyte) and rifamycin were from Pharmacia LKB Biotechnology. Formaldehyde, hexanal, sodium salt of NAD and NADH, DEAE-cellulose, AMP-Sepharose, and ADP-ribose were from Sigma Chemical Co. Acetaldehyde, propionaldehyde, butyraldehyde, glutaraldehyde, glycolaldehyde, 4-(dimethylamino)-cinnamaldehyde, benzaldehyde, *p*-nitrobenzaldehyde, *p*-meth-

† This work was supported in part by Grant AA05812 from the National Institute on Alcohol Abuse and Alcoholism. H.W. is a recipient of a Senior Scientist Award (AA00038) from the same Institute. Journal Paper 14 196 from the Purdue University Agriculture Experiment Station.

* Corresponding author: 1153 Biochemistry Department, Purdue University, West Lafayette, IN 47907-1153. Phone: 317-494-1650; Fax: 317-494-7897.

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

[1] Abbreviations: ALDH, aldehyde dehydrogenase; S74X, serine at position 74 changed to an alanine, cysteine, or threonine; DACA, 4-(dimethylamino)cinnamaldehyde.

0006-2960/94/0433-8955$04.50/0

oxybenzaldehyde, and *p*-nitrophenyl acetate were from Aldrich Chemical Co. L-[^{35}S]methionine and deoxyadenosine 5′-[α-^{35}S]thiotriphosphate triethylammonium salt were from Amersham. All other reagents were of analytical-grade purity.

Cells and Plasmids. *Escherichia coli* strain BL21 (DE3) pLysS (Studier & Moffatt, 1986) was a gift from Dr. W. Studier at the Brookhaven National Laboratory. The pT7 expression vector, a derivative of pT7-1 (Tabor & Richardson, 1985), was a gift from Dr. S. Tabor at Harvard University.

Mutagenesis. The mutations of native mitochondrial aldehyde dehydrogenase were created by oligonucleotide-directed mutagenesis. The codon for serine, TCG, at position 74 was altered to GCG, TGT, and ACG to produce mutants containing alanine, cysteine, and threonine, respectively, at this position. The alanine mutant was created by using the Muta-Gene kit from Bio-Rad (Sambrook *et al.*, 1989) whereas cysteine and threonine mutants were created by using Altered Sites *in vitro* Mutagenesis System (Promega Corp.). The methodology for mutagenesis was as described by the manufacturer with slight modifications. Essentially, the cDNA for native ALDH from pT7-7 plasmid (Zheng & Weiner, 1993) was excised by restriction enzyme digestion, subjected to electrophoresis for identification on the basis of size, and finally purified by using GENE CLEAN (BIO 101 Inc.). This cDNA was cloned into M13 vector (Sambrook *et al.*, 1989) for the creation of alanine mutant and into pSELECT phagemid (Promega Corp.) for the creation of cysteine and threonine mutants. The clones were transformed in JM 109 cells (Zheng & Weiner, 1993), and mutagenesis was conducted. The mutant colonies were selected by sequencing using the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977). After the identification of mutant colonies, 300 base pairs of DNA including the altered site were excised using the *Nde*I and *Nsi*I restriction enzymes and were exchanged with the exact-size piece of native cDNA from pT7-7 plasmid. The plasmids containing mutant cDNA were transformed in BL/21 cells containing chloramphenicol resistance pLysS plasmid. The mutation was again confirmed by double-stranded DNA sequencing of the pT7-7 plasmid from transformed BL/21 cells.

Purification of Native and Mutant Enzyme. The purification of native and mutant enzymes was conducted as described in Jeng and Weiner (1991) and Ghenbot and Weiner (1992), but the dialysis step to remove the protamine sulfate was replaced by a Sephadex G-25 step.

Enzyme Assays and Determination of Kinetic Constants. The aldehyde dehydrogenase activity was assayed by measuring the rate of increase in the fluorescence of NADH generated during the reaction at pH 7.4 in 100 mM sodium phosphate. Assays for the bisubstrate reaction kinetics for the native and the S74A were performed at different propionaldehyde and NAD concentrations. The K_m and V_{max} values for NAD were determined in the presence of 25 μM propionaldehyde whereas the K_m and V_{max} values for propionaldehyde were determined in the presence of 100 μM NAD for native and 5 mM NAD for mutant enzymes. The K_i values for ADP-ribose for the dehydrogenase reaction of the native and the S74A mutant were calculated using a Dixon plot.

The esterase activities of the recombinantly expressed native and mutant enzymes were determined by assaying the rate of formation of *p*-nitrophenol at 400 nM, in 100 mM sodium phosphate buffer (pH 7.4) with different concentrations of *p*-nitrophenyl acetate. The esterase activity in nmol min^{-1} (mg^{-1} of enzyme)$^{-1}$ was determined using 16×10^3 as the molar absorbance coefficient of *p*-nitrophenol at 400 nm. Equimolar concentration of bovine serum albumin was used as an enzyme substitute in control assays.

Isoelectric Point Determination. The native and mutant enzyme preparations were subjected to horizontal agarose isoelectric focusing as described by Pharamacia Chemical Co. using a 3–10 p*I* range gel. The isoelectric points were determined by calibrating the pH gradient of gel using p*I* standards.

Thermal Stability Test. Equimolar concentrations of enzyme (17 nM in 100 mM sodium phosphate buffer, pH 7.4) in 1.5-mL Eppendorf tubes were incubated at 50 °C for the desired time interval and then placed on ice for 5 min. Afterward, the tubes were left at room temperature for 15 min before assaying for ALDH activity at 25 °C. The native and mutant enzymes were assayed in the presence of 25 μM propionaldehyde and 0.5 and 5 mM NAD, respectively.

In Vivo Stability Test. The stability of native and S74A mutant was determined by pulse–chase experiments as described in Jeng and Weiner (1991).

Binding to AMP-Sepharose. AMP-Sepharose was regenerated as suggested by the manufacturer, poured as a 2.5-mL column, and equilibrated with degassed 10 mM sodium phosphate buffer (pH 6.1), 1 mM EDTA, and 0.025% 2-mercaptoethanol. Pure enzyme (approximately 275 μg) was dialyzed against 1 L of equilibration buffer for 2.5 h and centrifuged in a Eppendorf tube. The supernatant was assayed for ALDH activity and loaded on the AMP-Sepharose column at a flow rate of 0.2 mL/minute. When all the enzyme solution entered the gel, the flow was stopped for 15 min. The column was washed with 10 mL of equilibration buffer and then with 20 mL of 1 M sodium chloride in the equilibration buffer. Fractions (0.5 mL) were collected and assayed for activity in the presence of 25 μM propionaldehyde and 100 μM or 5 mM NAD for native and mutant enzymes, respectively.

Determination of Dissociation Constant (K_d) for NADH. K_d values for NADH were determined at 25 °C for the native and S74A mutant in the presence of 100 mM sodium phosphate buffer (pH 7.4) by measuring the fluorescence emission of unbound and bound NADH at 450 nM with excitation at 340 nm (Takahashi & Weiner, 1980) with a Hitachi 2000 fluorimeter.

Determination of Pre-Steady-State Burst Magnitude of the Dehydrogenase Reaction for Native and S74A. The pre-steady burst magnitude for ALDH activity of native and mutant enzymes was determined by the method of Takahashi and Weiner (1980) on a fluorimeter at 25 °C in the presence of 100 mM sodium phosphate buffer (pH 7.4), NAD (100 μM for native and 5 mM for S74A mutant), and 250 μM propionaldehyde. The enzyme concentrations were 0.14 and 0.2 μM for native and S74A mutant, respectively. Essentially, the base-line fluorescence was first obtained by incubating the enzyme and coenzyme in buffer then propionaldehyde was added to start the reaction. The tracing for the steady-state portion of the reaction was extrapolated to zero time to calculate the burst of NADH formation before the steady state was achieved.

RESULTS

Purification of Recombinantly Expressed Native and S74A Mutant of Rat Liver ALDH. We previously reported that the native enzyme could be purified by ion-exchange (DEAE-cellulose) and affinity chromatography on a hydroxyacetophenone column (Ghenbot & Weiner, 1992). G-25 Sephadex size exclusion was introduced to replace the dialysis step previously employed. Since the mutant enzyme was

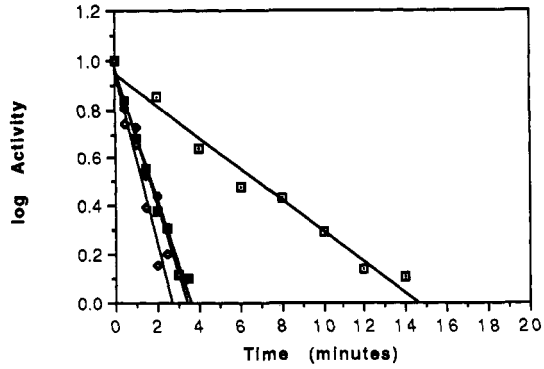

FIGURE 1: Relative thermal stability of purified native (⊡), S74A (♦), S74C (■), and S74T (◊) enzymes. Equimolar amounts of pure enzymes were incubated at 50 °C different time intervals and assayed for ALDH activity at 25 °C; reported in arbitrary units.

expected to have the same isoelectric point as native enzyme, the same purification scheme was employed. With mutant enzyme, it was not possible to detect catalytic activity with the standard assay (100 mM phosphate, pH 7.4, containing 100 μM NAD and 25 μM propionaldehyde). Thus, elution of the mutant enzyme from the columns was monitored by dot blotting (Farrés *et al.*, 1994). Once purified, it was possible to assay the mutant enzyme if the concentration of NAD was increased to 5 mM. The mutant enzyme was homogeneous on SDS–PAGE and had the same p*I* as that of native enzyme (5.1–5.2).

Stability of S74A. The effect of the point mutation on the stability of the enzyme was investigated both *in vivo* and *in vitro* with the purified enzyme. Pulse–chase analysis revealed that the mutant form had the same *in vivo* stability as that of the recombinantly expressed native enzyme. The purified enzyme was somewhat less stable at elevated temperatures. When the enzymes were incubated at 37 °C, both enzymes were stable toward denaturation. At 50 °C, though, the mutant had a half-life toward denaturation of 1 min compared to 4 min for the native enzyme (Figure 1). Thus the serine to alanine mutation affected both the thermostability of the enzyme as well as catalytic properties, which will be discussed below.

Bisubstrate Reaction Kinetics. The primary double reciprocal plots for the two substrate reaction are shown in Figure 2. These were converted into secondary plots (Figure 3) to determine the various kinetic constants, which are tabulated in Table 1 based on a reaction scheme showing the individual rate constants as presented in Figure 4. Two major differences between the native and the mutant enzyme became apparent. V_{max} was lowered by a factor of 11 while the K_m for NAD increased by a factor of nearly 200. In contrast, the K_m for propionaldehyde was the same for both enzyme forms. K_{ia}, the dissociation constant for NAD, increased 35-fold in the mutant. Thus the mutation at serine 74 affects NAD binding. For a two-substrate, ordered sequential reaction the ratio k_{cat}/K_m is k_1, the *on* velocity for the lead substrate. Since the K_m for aldehyde was not altered by the mutation, it is possible to suggest that the mutant enzyme still functioned with ordered binding. If this is the case, then the K_{ia} term decreased by a factor of 4×10^{-4} in the mutant and the rate constant for NAD dissociation, k_2, calculated from the K_{ia} term, also was found to have decreased in mutant. Thus the replacement of serine 74 caused the binding of NAD to be diminished, affecting both the *on* and *off* rates, k_1 and k_2, as well as the rate-limiting step.

Pre-Steady-State Burst Magnitude of NADH Formation. It has been demonstrated that there is a pre-steady-state burst

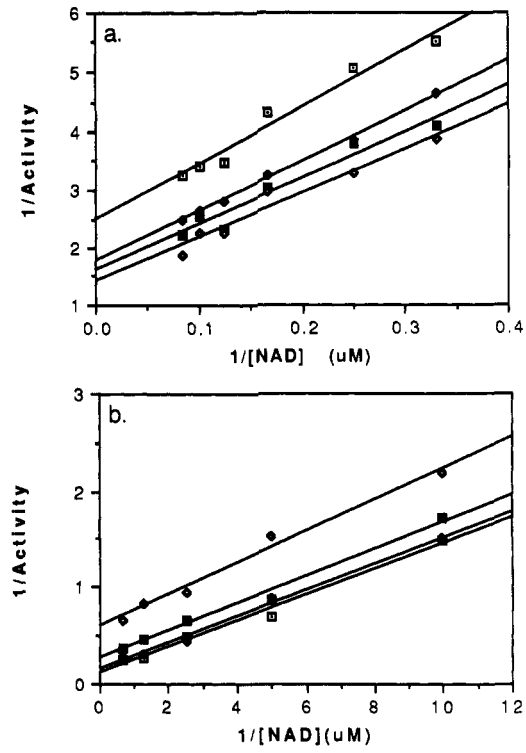

FIGURE 2: Primary plots of the bisubstrate reaction kinetics for the dehydrogenase reaction for native (a) and S74A (b) mutant enzymes. The propionaldehyde concentrations used for native enzymes were 0.3 (⊡), 0.5 (♦), 0.6 (■), and 0.7 (◊) μM, whereas for the S74A mutant they were 0.1 (◊), 0.3 (■), 0.6 (♦), and 0.7 (⊡) μM, respectively. Activity reported in arbitary units.

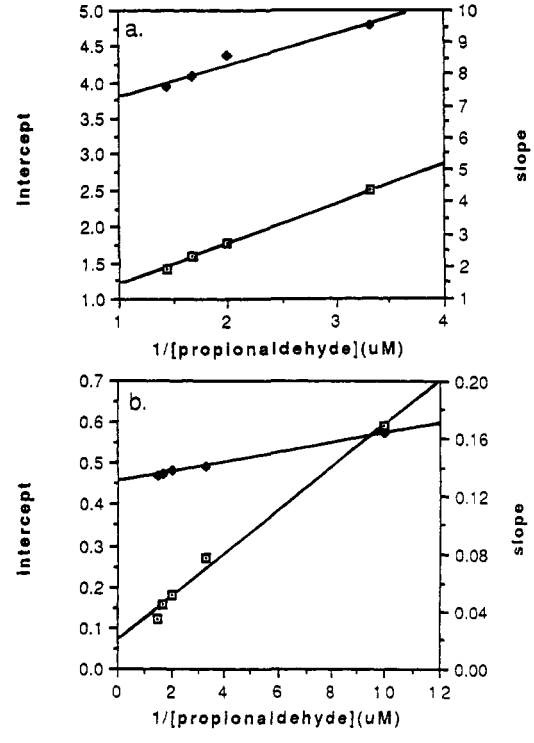

FIGURE 3: Secondary plots of bisubstrate reaction kinetics for the dehydrogenase reaction of native (a) and S74A (b) mutant enzymes obtained from the data in Figure 2, panels a and b, respectively. Intercepts (⊡) and slopes (♦).

of NADH formation with liver mitochondrial ALDHs (Weiner *et al.*, 1976). This has been interpreted to imply that the rate limiting step was k_7. The magnitude for the burst with the native enzyme was essentially 2 mol of NADH/mol of tetrameric enzyme, as shown in Figure 5. Essentially the

Table 1: Kinetic Parameters for the Dehydrogenase Reaction of Native and S74A Mutant Enzyme As Determined by Bisubstrate Reaction Kinetics

kinetic parameters[a]	native	S74A
V_{max} (nmol min^{-1} mg^{-1})	560	48
k_{cat} (min^{-1})	123	11
K_a (μM)	10	1900
K_b (μM)	0.8	0.8
K_{ia} (μM)	1.8	64
k_1 (μM^{-1} min^{-1})	12	0.005
k_2 (min^{-1})	22	0.3

[a] K_a is K_m for NAD; K_b is K_m for propionaldehyde; K_{ia} is K_d for NAD; k_1 is the *on* velocity for NAD (V_{max}/K_a) and k_2 is the *off* velocity of NAD as calculated from $K_{ia}k_1$.

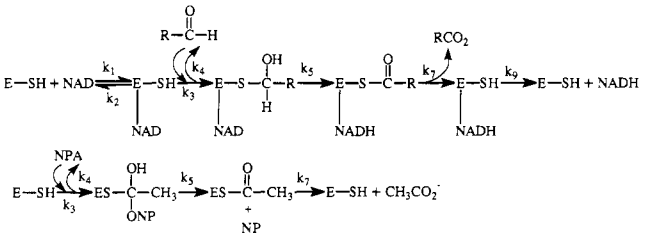

FIGURE 4: Schematic diagram of the postulated dehydrogenase and esterase reaction mechanism for aldehyde dehydrogenase (Weiner, 1979), where E represents enzyme, SH is the cysteine nucleophile, NPA is *p*-nitrophenyl acetate, and NP is nitrophenol. The acyl enzyme intermediate is formed at step 3, and the hydrolysis of the acyl enzyme intermediate, which is common for both the dehydrogenase and esterase reaction, occurs at step 7.

same value was reported by our laboratory for the horse and human enzymes (Weiner *et al.*, 1976; Farrés *et al.*, 1994). With the S74A mutant a burst was found but the magnitude decreased to just 1 mol/mol of enzyme. The fact that there still was a pre-steady-state burst of NADH implies that the rate-limiting step was still k_7. The significance of the change in magnitude of the burst will be discussed below.

Interaction of ALDH with an AMP-Sepharose and ADP-Ribose. Kinetic analysis showed impaired binding of NAD to the mutant enzyme. To determine if the alteration was related to the nicotinamide ring or another portion of the coenzyme, the interaction with NAD-like compounds was investigated. First, it was found that the mutant enzyme did not bind to an AMP affinity column while the recombinantly expressed native enzyme did. Next, the ability of ADP-ribose to act as a competitive inhibitor of the enzyme was investigated. The K_i value for the mutant enzyme was 6.8 mM compared to 0.32 mM for the native enzyme (data not shown). This 30-fold increase in K_i shows that the altered NAD binding was not due just to an interaction with the nicotinamide ring as we found to occur with an E487K mutant of the human liver enzyme (Farrés *et al.*, 1994).

Binding of NADH to the Enzymes. We have shown that one can use fluorescence to measure the binding of NADH to ALDH (Takahashi *et al.*, 1980). A titration curve and Scatchard plot are shown in Figure 6. This is similar to what we found with the horse liver enzyme (Takahashi *et al.*, 1980). The value for K_d was estimated to be 1 μM, while n was essentially 2, suggesting half-of-the-sites reactivity as we have found with other mammalian mitochondrial ALDHs (Takahashi & Weiner, 1980).

Unfortunately, the fluorescence of bound NADH was not enhanced very much with the mutant enzyme, as shown in Figure 6b. The small changes in fluorescence made it difficult to obtain accurate binding data with the mutant enzyme. Though there were only limited data, a least-squares fit (r^2 = 0.95) of the binding data allowed us to estimate that 2 mol of NADH still bind to the S74A mutant and that the K_d was approximately 7 μM. Thus, it appears that NADH, like NAD, binds more poorly to the mutant form than it did to the native enzyme.

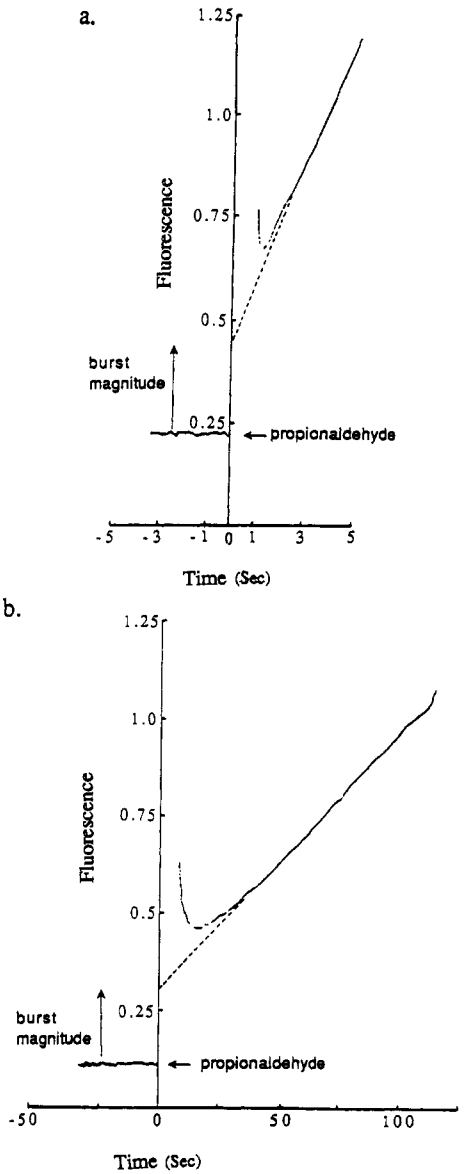

FIGURE 5: Pre-steady-state burst for the dehydrogenase reaction catalyzed by native and S74A mutant enzyme. The tracings (a, native; b, S74A) are for fluorescence measurement before (horizontal line) and after the inclusion of 250 μM propionaldehyde (at zero time) in the reaction mixture which contained enzyme (0.14 μM for native and 0.2 μM for S74A) and NAD (100 μM for native and 5 mM for S74A) in 100 mM sodium phosphate buffer (pH 7.4). The extrapolation of the steady-state tracing to time zero (dotted line) was used to measure the magnitude of the burst (moles of NADH produced per mole of tetrameric enzyme) before the steady-state reaction was attained. The fluorescence is presented in arbitrary units but was calibrated so 1 unit was equivalent to 1 μM NADH.

Substrate Specificity of the S74A Mutant. As shown in Table 1, the K_m for propionaldehyde was not significantly altered in the mutant enzyme, but the value for k_{cat} was. To determine if the mutation caused an alteration in the specificity constant (V/K_m) for different substrates, the kinetic constants for a limited number of commercially available aldehydes were determined (Table 2). For all, including both aromatic and aliphatic substrates, the K_m for the aldehyde was essentially the same for the native as with the mutant enzyme, while the V_{max} term was decreased in the mutant to under 10% of what

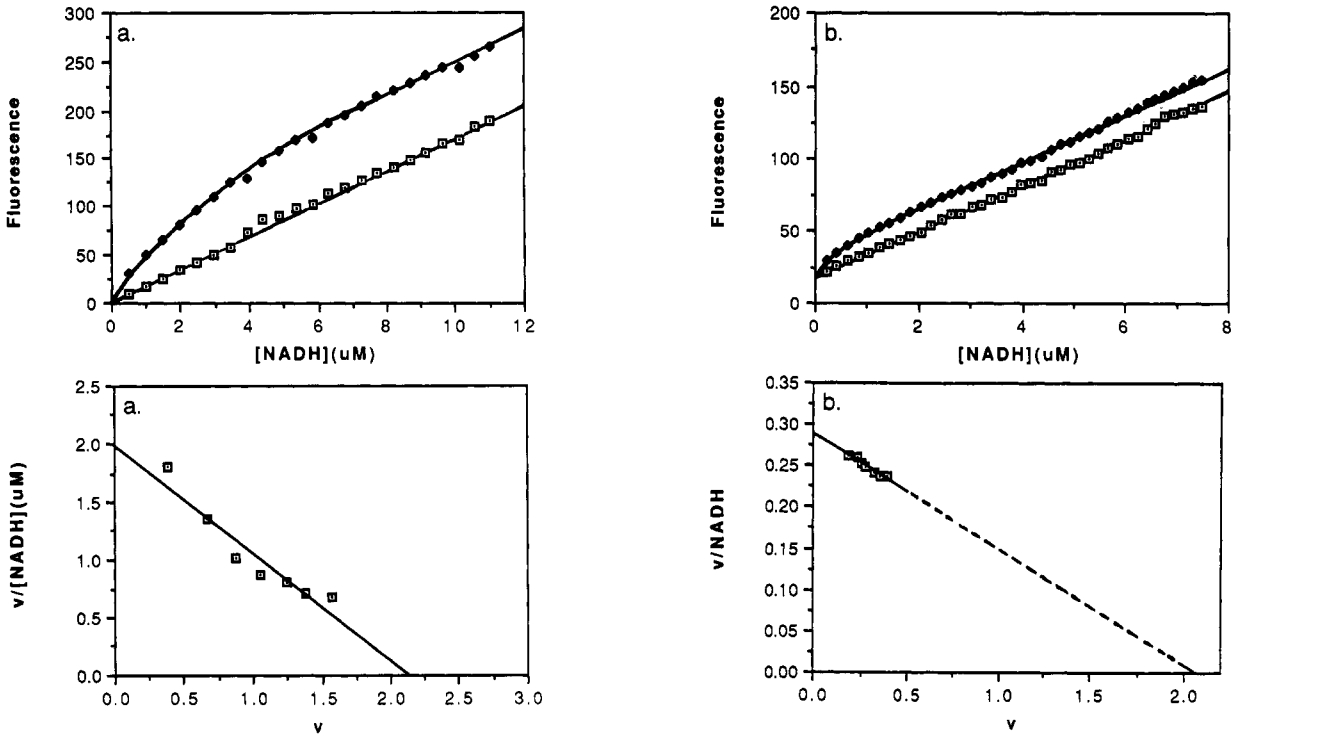

FIGURE 6: Fluorescence emission measurement of NADH (in arbitrary units) at 450 nm with excitation at 340 nm in the absence (□) and presence (♦) of 0.72 μM native (a) and 1.0 μM S74A mutant (b) enzyme. Lower two panels: The Scatchard plots corresponding to the fluorescence enhancement data of the native and the mutant enzymes, respectively, showing the stoichiometry of NADH binding, v, per mole of tetramic enzyme.

Table 2: K_m and V_{max} Values for Dehydrogenase Reactions As Determined by Lineweaver–Burk Plots for the Native and S74A Mutants

substrate	enzyme	K_m[a]	V_{max}[b]	V_{max}[c] (%)	V_{max}/K_m
formaldehyde	native	178	1360	0.5	7.8
	S74A	700	7		0.01
acetaldehyde	native	0.4	561	7	1400
	S74A	2.7	37		14
propionaldehyde	native	0.7	495	8	617
	S74A	0.9	38		42
butyraldehyde	native	0.7	831	6	1190
	S74A	2	52		26
hexaldehyde	native	0.8	1220	7	1530
	S74A	2.4	81		34
DACA	native	0.1	120	4	122
	S74A	0.3	5		17
benzaldehyde	native	0.1	72	10	720
	S74A	0.2	7		35
p-nitrobenzaldehyde	native	0.1	275	9	2750
	S74A	0.4	25		63
p-methoxybenzaldehyde	native	0.1	15	3	150
	S74A	0.4	0.5		1.3
glutaraldehyde	native	0.9	566	1	630
	S74A	3	7		3.3
glycolaldehyde	native	38	1690	1	44
	S74A	77	18		0.2

[a] K_m for aldehydes in μM. [b] V_{max} in terms of nmol min^{-1} (mg of enzyme)$^{-1}$. [c] V_{max} of S74A as a percent of native.

Table 3: K_m and V_{max} Values for NAD and Propionaldehyde As Determined by Lineweaver–Burk Plots for the Native and S74 Mutant Enzymes

enzyme	substrate	K_m (μM)	V_{max}[a]	rel V_{max} (%)
native	NAD	20	510	100
S74A		2100	45	9
S74C		1400	23	5
S74T		2000	16	3
native	propionaldehyde	0.7	495	100
S74A		0.9	38	8
S74C		1	20	4
S74T		1.6	19	4

[a] V_{max} in terms of nmol min^{-1} mg^{-1}.

was found with the native enzyme. Even with DACA, the substrate originally used to identify serine 74 as a possible component of the active site (Loomes *et al.*, 1990), the mutant retained 4% of native activity.

Substitution of Serine by Cysteine or Threonine. Since S74A was still an active enzyme, we can conclude that serine does not function as the essential catalytic residue. The importance of having a potential nucleophilic residue at that position was explored by making the cysteine and the threonine mutants. Both of these mutants were purified as described for the alanine mutant. Each proved to be active when assayed with a high concentration of NAD. The activity, though, was similar to that of the alanine mutant and not to that of the native enzyme. The K_m for propionaldehyde was unchanged from that found with the native enzyme. These data are tabulated in Table 3. All the S74 mutants examined had similar pH–activity profiles which were like that of the native enzyme. All the 74-mutants had the same thermal stability at 50 °C, and none bound to an AMP-Sepharose column.

Esterase Activity of Native and Mutant Forms of ALDH. We have shown that ALDH can hydrolyze *p*-nitrophenyl acetate and that the reaction was stimulated by the presence of NAD or NADH (Feldman & Weiner, 1972; Takahashi & Weiner, 1981). The ability of the mutant forms of ALDH to hydrolyze the ester was determined along with the concentration of coenzyme necessary to stimulate the reaction. The K_m for substrate in the absence of coenzyme was found to be 66 μM for the recombinantly expressed native enzyme.

Table 4: Specific Activity of the Esterase Reaction for the Native and S74A Mutants in the Absence and Presence of Coenzyme

enzyme	coenzyme	sp act.[a]	sp act. (%)	coenzyme concn[b]
native	none	417	100	
S74A		11	3	
S74C		22	5	
S74T		13	3	
native	NAD	1260	100	50 μM
S74A		101	8	1.7 mM
S74C		184	15	1.1 mM
S74T		108	9	1.9 mM
native	NADH	1690	100	11 μM
S74A		200	12	260 μM
S74C		400	24	170 μM
S74T		264	16	270 μM

[a] Specific activity in nmol min^{-1} (mg of enzyme)$^{-1}$. [b] Coenzyme concentration needed to raise the specific activity to its half-maximum.

With 800 μM substrate, the equivalent of a V_{max} assay, it was found that all three mutant enzymes were active, but they possessed less than 5% of the activity of the native enzyme. Increasing the concentration of ester did not cause an increased rate of reaction. It was not possible to determine an accurate K_m value with an enzyme of such low specific activity due to the spontaneous hydrolysis of *p*-nitrophenyl acetate and the potential nonspecific hydrolysis caused by adding a much higher concentration of protein to the reaction. Thus all reactions were performed with 800 μM *p*-nitrophenyl acetate.

The addition of coenzyme stimulated the esterase activity of the mutant forms. The concentration of NAD or NADH required for half-maximal stimulation is presented in Table 4. Approximately 20 times more coenzyme was required to obtain a 50% stimulation of activity with the mutants compared to the native enzyme. This is consistent with the poor binding of coenzyme caused by the replacement of serine 74.

DISCUSSION

Even before the primary structures of aldehyde dehydrogenases were known, it was observed that thiol-directed cysteine reagents would cause the inactivation of the enzyme (Hempel & Pietruszko, 1981). It was postulated that the enzyme oxidized aldehydes in a manner analogous to that of glyceraldehyde-3-phoshphate dehydrogenase (Feldman & Weiner, 1972). That is, a cysteine forms a hemiacetal intermediate which is oxidized to a thioester. This laboratory suggested, based upon chemical modification work, that cysteine 49 was involved as the active nucleophile (Tu & Weiner, 1988), while data from others suggested that cysteine 302 was the essential residue (Blatter *et al.*, 1990). Now that more than a dozen different ALDHs from diverse organisms have been sequenced, it is apparent that the only nucleophilic residue that is completely conserved is cysteine 302 (Hempel *et al.*, 1993). Our laboratory has cloned and expressed rat and human mitochondrial ALDH and has used site-directed mutagenesis to probe the role of the various cysteines and found only the one at position 302 could be essential (Weiner *et al.*, 1991).

The oxidation of an aldehyde to an acid need not proceed though a thioacetal intermediate, but could involve an acetal. If this be the case, the intermediate formed would be an O-ester with the enzyme rather than a thioester, and isolation of a serine ester adduct during the oxidation of DACA by sheep liver cytosolic ALDH has been reported (Loomes *et al.*, 1990). The yield of the isolated adduct was very low. Yet, this alone does not negate the possibility of serine being involved as an essential component of the active site. When one compares the sequences of all known ALDHs (Hempel *et al.*, 1993), it is found that the residue at position 74 is not conserved among the enzymes. To try to determine whether or not the serine at this position functions as the essential nucleophile, we performed site-directed mutagenesis to change the residue into others.

The various S74-mutants of rat liver mitochondrial ALDH were all found to be catalytically active, but their k_{cat} value was decreased more than 10-fold. Thus, even with DACA, the substrate employed when the sheep liver enzyme was investigated, all the recombinantly expressed mutant enzymes were still active. Hence, it can be concluded that serine 74 is not a mandatory residue in the active site of ALDH. It was totally unexpected, then, to find that replacing the serine with other residues caused such drastic changes to occur in the catalytic properities of the enzyme. Not only did the value of k_{cat} decrease 10-fold, but the K_m and K_d for NAD increased dramatically.

Inasmuch as the three-dimensional structure has not yet been determined, it is impossible to state how a change at position 74 affects the binding of coenzyme and the K_m for NAD. It was shown that the threonine or cysteine replacement for serine did not restore the tight NAD binding ability of the enzyme. By comparing the typical structures of an NAD binding domain to the known sequences of ALDHs, Hempel suggested that the coenzyme binding pocket, GXGXXG, should include residues around positions 295–327 (Hempel *et al.*, 1993). We recently found that converting glutamate 487 to a lysine also caused a dramatic effect on the K_m and K_d for NAD with both human and rat liver mitochondrial ALDHs (Farrés *et al.*, 1994). It is possible that the "Rossmann" binding domain includes residues from both the extreme C-terminal end of the 500 amino acid enzyme and residue 74. We cannot say whether or not the serine directly interacts with coenzyme. If it did, it most likely would provide a hydrogen bond to the phosphate or ribose, similar to what has been shown to exist in other proteins (Wierenga & Hol, 1983; Wilson *et al.*, 1983). It is possible that a minor alteration in the conformation of the protein at position 74 affected the chain near the actual binding site for NAD, for neither cysteine nor threonine could restore native-like properties to the enzyme.

The loss of serine appears to do more than just affect NAD binding to the enzyme, for the V_{max} values for both the dehydrogenase and the esterase reaction were decreased. We have previously shown that the presence of NAD increases the nucleophilicity of the active site residue (Takahashi & Weiner, 1981), now known to be cysteine 302. It has been argued that the rate-limiting step for the oxidation of aldehydes by the enzyme is deacylation, k_7 (Weiner *et al.*, 1976). Finding that the velocity was a function of the substrate being oxidized (Table 2) supports this notion and not the alternative, that coenzyme dissociation was rate limiting (Vallari & Pietruszko, 1981). In the S74A mutant V_{max} decreased by a factor greater than 10. Perhaps the altered binding of NAD somehow affects the deacylation step, which may occur while NADH is still bound to the enzyme, as illustrated in Figure 4. The rate of hydrolysis of *p*-nitrophenyl acetate was a factor of 20 lower with the S74A mutant compared to native enzyme, showing that the low turnover number with the mutant during the dehydrogenase reaction was not related to aldehyde oxidation or coenzyme dissociation. This leads us to suggest that replacement of serine 74 affects the catalytic properties of the enzyme even if NAD(H) is not present. Finding that the

thermostability of the mutants was decreased compared to the native enzyme is consistent with the notion that the mutation caused an alteration in the enzyme.

A pre-steady-state burst of NADH formation was observed during the oxidation of aldehyde by both the recombinantly-expressed native enzyme, shown in Figure 6, as was previously observed with this enzyme (Farrés *et al.*, 1994) and the S74A mutant. The magnitude of the burst was 1 mol NADH/mol of tetrameric mutant enzyme rather than 2, as found with the native enzyme. [See Takahashi and Weiner (1980) for a discussion of the half-of-the-sites reactivity of ALDH]. The fact that there still was a burst implies that k_7 is still the rate-limiting step for the mutant. To explain the burst magnitude it is necessary to compare k_3 and k_7. The burst magnitude, (Bender *et al.*,1967) is given by

$$\frac{E_T}{[1 + (k_7/k_3)]^2}$$

To find a burst magnitude equivalent to the number of functioning active sites (E_T), the ratio of k_7/k_3 would have to be very small, at least less than 0.05. To obtain a value of 1, as found with the mutant, where NADH binding studies showed it to have the same number of functioning active sites as did native enzyme, the ratio k_7/k_3 would have to be around 0.4. If the step governing k_{cat} remained the same with the mutant form, the value of k_7 would have decreased by a factor of 10 in the mutant. If k_3 had the same value in both the mutant and native enyzme, the ratio of k_7/k_3 in the mutant would have actually become lower not higher. Thus the value of k_3 must have also decreased. Evidence to support this suggestion comes from the esterase reaction. We previously proposed, at least for the horse liver mitochondrial enzyme, that k_3 is the rate-limiting step for esterase reaction (Weiner *et al.*, 1976). The data in Table 4 show that the mutant form had just 2.5% of the specific activity of the native enzyme in the absence of added coenzyme. Thus the value for k_3 may have decreased 40-fold as a result of the S74A mutation. In the presence of either NAD or NADH the alanine mutant had around 10% of the activity of the native enzyme. Under those assay conditions the value of k_3 was decreased by a factor of 10. Thus, the loss of the serine residue which resulted in altered coenzyme binding affects a number of rate constants. It is possible that the presence of the serine acts to stabilize the overall transition state and this stabilization cannot be restored by a threonine or cysteine residue.

It is not apparent why the investigators who studied the sheep liver cytosolic ALDH found that DACA labeled serine 74. It is possible that this was due to a transesterification reaction that occurred while they were isolating the labeled enzyme as was suggested by the authors (Loomes *et al.*, 1990). The data presented here clearly show that this serine is not a mandatory residue for catalytic activity, but a modification to the hydroxyl causes the enzyme to have altered binding properties toward coenzyme and a 10-fold decrease in its specific activity.

REFERENCES

Abriola, D. P., Fields, R., Stein, S., Mackerell, A. D. Jr., & Pietruszko, R. (1987) *Biochemistry 26*, 5679–5684.

Bender, M. L., Kezdy, F. J., & Wedler, F. C. (1967) *J. Chem. Educ. 44*, 84–88.

Blatter, E. E., Tasayco, J. M. L., Prestwitch, G., & Pietruszko, R. (1990) *Biochem. J. 272*, 351–358.

Bradbury, S. L., & Jakoby, W. B. (1971) *J. Biol. Chem. 246*, 6929–6932.

Farrés, J., Guan, K.-L., & Weiner, H. (1989) *Eur. J. Biochem. 180*, 67–74.

Farrés, J., Wang, X.-P., Takahashi, K., Cunningham, S. J., Wang, T. T. Y., & Weiner, H. (1994) *J. Biol. Chem. 269*, 13854–13860.

Feldman, R. I., & Weiner, H. (1972) *J. Biol. Chem. 247*, 267–272.

Ghenbot, G., & Weiner, H. (1992) *Protein Exp. Purif. 3*, 470–478.

Hempel, J., & Pietruszko, R. (1981) *J. Biol. Chem. 256*, 10889–10896.

Hempel, J., Kaiser, R., & Jörnvall, H. (1985) *Eur. J. Biochem. 153*, 13–18.

Hempel, J., Nicholas, H., & Lindahl, R. (1993) *Protein Sci. 2*, 1890–1990.

Jeng, J., & Weiner, H. (1991) *Arch. Biochem. Biophys. 289*, 214–222.

Kitson, T. M., Hill, J. P., & Midwinter, G. G. (1991) *Biochem. J. 275*, 207–210.

Loomes, K. M., Midwinter, G. G., Blackwell, L. F., & Buckley, P. D. (1990) *Biochemistry 29*, 2070–2075.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, Cold Spring Harbor, NY.

Sanger, F. , Nicklen, S., & Conlson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Studier, G. W., & Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113–130.

Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074–1078.

Takahashi, K., & Weiner, H. (1980) *J. Biol. Chem. 255*, 8206–8209.

Takahashi, K., & Weiner, H. (1981) *Biochemistry 20*, 2720–2726.

Takahashi, K., Weiner, H., & Hu, J. H. J. (1980). *Arch. Biochem. Biophys. 205*, 571–578.

Tu, G.-C., & Weiner, H. (1988) *J. Biol. Chem. 263*, 1218–1222.

Vallari, R. C., & Pietruszko, R. (1981) *Arch. Biochem. Biophys. 212*, 9–13.

Weiner, H. (1979) in *Biochemistry and Pharmacology of Ethanol* (Majchrowicz, E., & Noble, E. P., Eds.) pp 107–124, Plenum, New York.

Weiner, H., King, P., Hu, J. H. H., & Bench, W. R. (1974) in *Alcohol and Aldehyde Metabolizing Systems* (Thurman, R. G., Yonetani, T., Williams, J. R., & Chance, B., Eds.) Vol. I, pp 101–113, Academic Press, New York.

Weiner, H., Hu, J. H. J., & Sanny, C. G. (1976) *J. Biol. Chem. 251*, 3853–3855.

Weiner, H., Farrés, J., Wang, T. T. Y., Cunningham, S. J., Zheng, C.-F., & Ghenbot, G. (1991) in *Enzymology and Molecular Biology of Carbonyl Metabolism 3* (Weiner, H., Wermuth, B., & Crabb, D. W., Eds.) pp 13–17, Plenum Press, New York.

Wierrenga, R. K., & Hol, W. G. J. (1983) *Nature 302*, 842–844.

Wilson, D. K., Bohren, K. M., Gabbay, K. H., & Quiocho, F. A. (1992) *Science 257*, 81–84.

Zheng, C.-F., & Weiner, H. (1993) *Arch. Biochem. Biophys. 305*, 460–466.