Evidence for Two Distinct Active Sites on Aldehyde Dehydrogenase*

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Aldehyde dehydrogenase can catalyze the hydrolysis of esters such as \( p \)-nitrophenyl acetate as well as oxidize aldehydes to acids. It has not been proven unequivocally that the two reactions occur at the same active site. In the accompanying paper (Tu, G. C., and Weiner, H. (1988) J. Biol. Chem. 263, 1212-1217) evidence was presented which showed that cysteine at position 49 was at the active site for the dehydrogenase reaction. Evidence also was presented which showed that cysteine located at position 162 was susceptible to modification by N-ethylmaleimide. It was shown here that the two activities of the enzyme can be differently protected from inactivation by substrate analogs. Furthermore, aldehydes were found to be poor inhibitors against the esterase reaction while ester was a good inhibitor against the dehydrogenase reaction. In addition, it was possible to modify cysteine 49 with N-ethylmaleimide but not find inhibition of the esterase reactivity until cysteine 162 was modified. It appears that horse liver aldehyde dehydrogenase has two separate active sites per subunit. The data fit a model where ester can be hydrolyzed at both sites but that aldehyde oxidation occurred only at position 49.

Horse liver aldehyde dehydrogenase like all mammalian aldehyde dehydrogenases can catalyze the NAD-dependent oxidation of various aldehydes as well as the hydrolysis of activated esters such as nitrophenyl acetate (1). It was originally proposed by Feldman and Weiner (2) that both reactions occurred at the same reactive site. Although not proved at the time it was suggested that the active site possessed a cysteine residue which acted as a nucleophile attacking the carbonyl of either aldehyde or ester. With the former, a thiohemiacetal intermediate (I) would be formed prior to the acyl intermediate (II). The acyl enzyme intermediate would be formed directly from attack of the nucleophile on the ester as illustrated:

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\begin{align*}
\text{H} & \quad E'\text{SH} + \text{CH}_3\text{CO}^+ \\
\text{CH}_3\text{CHO} & \quad E'\text{SH} + \text{CH}_3\text{CO}_2^-
\end{align*}
\]

\( E'\text{SH} \) is an NAD(H) bound subunit of aldehyde dehydrogenase, NPA is \( p \)-nitrophenyl acetate, and NP is nitrophenol. It was shown with horse liver mitochondrial enzyme that the rate-limiting step for the dehydrogenase reaction was deacylation while it was acylation step for the esterase reaction in the absence of NAD (3) but was deacylation in its presence (4).

Although both the esterase and dehydrogenase reaction could ultimately form a thioacyl intermediate it was never proven that the same nucleophilic amino acid was responsible for both reactions. A number of investigators have presented cogent arguments favoring only one active site for both reactions while others present evidence supporting separate sites (5-10). Duncan (11) summarized most of the published kinetic data and concluded that a one-site model would best fit the data. The major non-kinetic evidence supporting the one-site model was that if one incubated the enzyme with an activated ester the acyl intermediate formed could be reduced by NADH to the corresponding aldehyde (5, 9, 10). This observation led to the suggestion that the esterase site was not only in proximity to the nicotinamide ring of NADH but was in the proper orientation so the acyl intermediate can be reduced.

Chemical modification studies, although, produce data which can best be interpreted as implying that separate active sites for the esterase and dehydrogenase exist (6, 12, 13). Inasmuch as the actual nucleophilic amino acid in the active site had not been identified unequivocally we undertook a study to determine if the active site of horse liver mitochondrial aldehyde dehydrogenase did indeed possess a cysteine residue as indicated by spectroscopic studies (14). It was reported in the accompanying paper (15) that cysteine residue at position 49 (corresponding to the sequence of the human enzyme (16)) was responsible for the dehydrogenase reaction. Evidence was presented to show that cysteine at position 162 could also be protected from modifications by aldehyde and a substrate analog making it appear that this cysteine had properties of an active site residue. We wanted then to determine if the esterase and dehydrogenase reactions were occurring at the same active site. In this paper we present evidence to show that separate sites can exist for the two reactions and that cysteine 162 may be the second site.

EXPERIMENTAL PROCEDURES

Materials

Propionaldehyde, \( p \)-nitrophenyl acetate, and phenyl benzoate were obtained from Aldrich. DL-Glyceraldehyde, NAD, N-ethylmaleimide, phenylmethanesulfonyl fluoride, and chloral hydrate were purchased from Sigma. Other chemicals available from commercial sources were of reagent grade.

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Distilled aldehyde-free water was used to prepare solutions. Buffers were from sodium salts.

Preparation of Horse Liver Mitochondrial Aldehyde Dehydrogenase

The preparation of the enzyme was as described in the accompanying paper (15).

Activity Assay

Both dehydrogenase and esterase activity assays were performed in 0.1 M phosphate buffer, pH 7.4, containing 500 μM NAD at 25 °C. Reactions were initiated by the addition of substrate to the enzyme solution and the changes either in fluorescence or in absorbance were recorded as a function of time (2, 17). The dehydrogenase reaction was assayed by monitoring fluorometrically the formation of NADH. The esterase reaction velocity was determined by assaying for p-nitrophenyl acetate in the absence of the enzyme. The reaction was much slower than was the rate of loss of dehydrogenase reactivity. Furthermore, although chloral hydrate, glyceraldehyde, and 125 mM p-nitrophenyl acetate was assayed by monitoring fluorometrically the formation of NADH.

Inhibition Kinetics

Substrates as Competitive Inhibitors—In order to observe the effect on the esterase on the dehydrogenase reaction, the concentration of aldehyde was kept at two times its K_m value, and the concentration of ester, which was considered as the competitive inhibitor, was varied from 0- to 20-fold of its K_m value. The reactions were initiated by the addition of the enzyme. The activity assay was performed as mentioned above and data graphed as a Dixon plot. The experiments were repeated using aldehyde as the inhibitor of the esterase reaction.

Nonsubstrate as Competitive Inhibitors—The nonsubstrates at various concentrations were first mixed with the enzyme in the presence of 500 μM NAD. The reaction was initiated by the addition of the substrate and the data graphed as a Dixon plot.

Chemical Modification

After removing mercaptoethanol and glycerin, used to protect the enzyme activity during storage (15), by gel filtration the enzyme was incubated with modifier in the presence of 500 μM NAD and 2 mM chloral hydrate in 0.1 M phosphate buffer, pH 7.5, at 25 °C. At zero time, NEM was added at a final concentration of 200 μM. Aliquots were removed for assay at the different intervals. The activity remaining was determined from the average value of six experiments: O, dehydrogenase activity; Δ and A, esterase activity remaining with and without chloral hydrate protection, respectively.

RESULTS

Irreversible Inhibitors of Activity—In the accompanying paper (15) it was shown that NEM could inhibit the dehydrogenase reaction catalyzed by horse liver mitochondrial aldehyde dehydrogenase. Similar studies were performed while assaying for the ability of the enzyme to hydrolyze p-nitrophenyl acetate. It was found that the rate of loss of esterase activity was much slower than was the rate of loss of dehydrogenase reactivity. Furthermore, although chloral hydrate, a substrate competitive inhibitor, protected the enzyme from inactivation by NEM 1 it was found that chloral hydrate afforded more protection against loss of esterase activity than the rate of loss of dehydrogenase activity. This data is summarized in Fig. 1.

The classical serine protease inhibitor phenylmethanesulfonyl fluoride also was tested as an inhibitor of the two reactions catalyzed by aldehyde dehydrogenase. The reagent at a fixed concentration proved to inhibit the esterase reaction to a greater extent than it inhibited the dehydrogenase reaction. As can be observed from the data presented in Fig. 2 some of the dehydrogenase reaction was restored as a function of time suggesting that the covalent bond was less stable than that found with serine in chymotrypsin or in other serine proteases (18). A thiol sulfonate ester linkage would be less stable than one formed with serine as demonstrated with papain (19).

Substrates as Competitive Inhibitors—If both reactions catalyzed by the enzyme were occurring at the same active site, the presence of one substrate should act as a competitive inhibitor against the other substrate. This test was applied and results indicated that p-nitrophenyl acetate was a good competitive inhibitor of aldehyde oxidation, as shown by a Dixon plot in Fig. 3. The K_m value was 5 μM which was virtually identical to its K_m value (10 μM). In Fig. 4, A and B, are presented data for two aldehydes, propionaldehyde, and DL-glyceraldehyde. Each aldehyde produced a biphasic inhibition.

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1 The abbreviation used is: NEM, N-ethylmaleimide.
Two Active Sites on Aldehyde Dehydrogenase

pattern when tested as inhibitors of the esterase activity. Table I summarizes the data.

Mg²⁺ Ion Effects on Inhibition—It had been previously shown that Mg²⁺ ion stimulates dehydrogenase activity 2-fold but did not alter the rate of esterase reaction (4, 20). A detailed explanation for the Mg²⁺ ion effect on the dehydrogenase reaction has been presented; the arguments include dissociation of tetrameric horse liver enzyme to a pair of dimers and an increase in the number of functioning active sites (20). In the absence of the metal the enzyme functioned with half of the site reactivity while in the presence of Mg²⁺ ions it functioned with full site reactivity when the dehydrogenase reaction was monitored (20, 21). It was now found that in the presence of Mg²⁺ ions neither propionaldehyde nor glyceraldehyde inhibited the esterase reaction. The converse was not found: p-nitrophenyl acetate was still a good inhibitor against the aldehyde dehydrogenase reaction. (K, was the same in the absence of Mg²⁺ ions.) Thus, Mg²⁺ ions appeared to affect each reaction differently.

The inhibition experiments were repeated using saturating concentration of substrate to determine whether the presence of one substrate would affect the Vₘₐₓ for the other. If totally separate sites existed, one would expect the enzyme to produce products from one reaction at a rate independent of the presence of the other substrate. If although, a common active site was involved the rate of product formation for each reaction would be half of the Vₘₐₓ found in the absence of the other substrates.

The experiments were performed in the presence and absence of Mg²⁺ ions. Table II presents a summary of the data. In the absence of Mg²⁺ ions each substrate inhibited the Vₘₐₓ of the other reaction. This implies that there might be some overlap in the binding domain or that there was a common active site component necessary for both reactions. Different results were obtained in the presence of Mg²⁺ ions. Ester again inhibited the dehydrogenase reaction but aldehyde did not affect the esterase reaction. This was similar to the results found when sub Vₘₐₓ assay conditions were employed. The combined results indicate that in the presence of Mg²⁺ ions, separate active sites function.

Analogs as Inhibitors—Two substrate analogs were also investigated as potential inhibitors of both the esterase and dehydrogenase reaction. Phenyl benzoate and chloral hydrate each proved to be competitive inhibitors against both reactions. Different results were obtained in the presence of Mg²⁺ ions. This data is summarized in Table III.

Labeling of Cysteine 162—In the accompanying paper (15)

![Fig. 3. A Dixon plot for the inhibition of dehydrogenase reaction by p-nitrophenyl acetate.](image)

![Fig. 4. Dixon plots for the inhibitions of esterase reaction by propionaldehyde (A) or glyceraldehyde (B).](image)

**Table I**

| [Inhibitor] µM | [Substrate] µM | K, µM |
|----------------|----------------|-------|
| p-Nitrophenyl acetate (0-20) | Glyceraldehyde (400; 1000) | 10.6  |
| Propionaldehyde (0-8) | p-Nitrophenyl acetate (1) | 0.2  |
| Glyceraldehyde (20; 50) | p-Nitrophenyl acetate (2) | 200  |
| Glyceraldehyde (4000) | p-Nitrophenyl acetate (2) | 4200 |

* Inhibitor range and substrate concentrations.

* K, for the compounds when functioning as substrates for the esterase and dehydrogenase reaction, respectively.

**Table II**

| Inhibitor | Mg²⁺ | Velocity (nmol/min) |
|-----------|------|---------------------|
| Propionaldehyde | 1.17 | 2.57 |
| Propionaldehyde | 0.61 | 1.51 |
| p-Nitrophenyl acetate | 2.5 | 2.97 |
| Propionaldehyde | 2.5 | 2.60 |
| p-Nitrophenyl acetate | 2.5 | 0.93 |

Effect of one reaction on the velocity of the other reaction under Vₘₐₓ conditions in the presence or absence of added Mg²⁺ ions

The concentration of propionaldehyde as substrate or inhibitor was 3.0 µM (15 × K, value) while that of p-nitrophenyl acetate was 125 µM (12.5 × K, value).
TABLE III

Effects of substrate analogs as inhibitors on both dehydrogenase and esterase activities of horse liver aldehyde dehydrogenase

Both reactions performed in 0.1 M phosphate at pH 7.4 in the presence of 500 μM NAD.

| [Inhibitor] μM | [Substrate] μM | Kᵢ μM |
|---------------|---------------|-------|
| Phenyl benzoate | Glyceraldehyde | 15    |
| (0-50)        | (400; 1,000)  |       |
| Phenyl benzoate | p-Nitrophenyl acetate | 75    |
| (0-350)       | (20; 50)      |       |
| Chloral hydrate | Glyceraldehyde | 3     |
| (0-20)        | (400; 1000)   |       |
| Chloral hydrate | p-Nitrophenyl acetate | 15    |
| (0-100)       | (20; 50)      |       |

*Inhibitor range and substrate concentrations.

TABLE IV

Relationship between NEM incorporation and loss of enzyme activities

Aldehyde dehydrogenase was first incubated as described in the legend of Fig. 1 for 15 min and then [14C]NEM was added at a final concentration of 20 μM. At indicated times both activities remaining were determined and aliquots were removed to be subjected to trypic hydrolysis and peptide mapping according to the procedures in the accompanying paper (15). Radioactivity was determined in 100-μl samples from each fraction obtained by high performance liquid chromatography separation.

| Incubation time | Activity remaining | cpm × 10⁷ |
|-----------------|--------------------|------------|
|                 | Dehydrogenase | Esterase | Cys-49 | Cys-162 |
| h               | %        | %        |        |         |
| 0               | 100      | 100      | 0      | 0       |
| 0.25            | 65       | 100      | 0      | 0       |
| 1.50            | 47       | 95       | 1.9    | 0.22    |
| 17.5            | 0        | 55       | 4.9    | 6.3     |

*Counts found in peak B and C corresponded to the peptides containing cysteine 49 and 162, respectively. See the accompanying paper (15).

The chemical modification data presented in this study can best be interpreted as showing that two classes of active sites exist. First it was possible to differentially inhibit the two reactions. Reagents other than NEM also differentially inhibited the two reactions catalyzed by aldehyde dehydrogenase (6, 12, 13). In a recent publication (13) we presented data to show that disulfiram, which was shown to attack cysteine residue 302 in human cytosolic aldehyde dehydrogenase (22), inhibited the enzyme in such a manner that the rate of inactivation of dehydrogenase was more rapid than was the rate of inactivation of the esterase activity. Furthermore, as shown in this study, chloral hydrate afforded a different degree of protection against NEM inhibition of each separate reaction. Similar results were obtained using chloral hydrate to protect against diethyl pyrocarbonate (a histidine reagent) inactivation of the enzyme (23). In that study it was found that chloral hydrate protected the enzyme against inactivation of dehydrogenase activity but afforded only partial protection against loss of esterase reaction (24).

It is possible to propose a two-active site model: one site for dehydrogenase, the other for the esterase reaction. In the absence of Mg²⁺ ions, ester could strongly bind to both sites (Kᵢ = Kᵢᵥ values), so it appeared that aldehyde could bind to two sites of the dehydrogenase reaction; aldehyde could strongly bind to the dehydrogenase site (Kᵢᵥ = Kᵢᵥ) but weakly to the other site (Kᵢᵥ = 20-50 × Kᵢᵥ). Thus, aldehyde appeared to be a poor inhibitor of the esterase reaction. In the presence of Mg²⁺ ions ester could still bind strongly to both sites, while aldehyde could no longer bind to the ester site; thus ester still remained a good inhibitor of the dehydrogenase reaction, but now aldehyde did not inhibit the esterase reaction. Finding that both chloral hydrate and phenyl benzoate each had two separate Kᵢᵥ values for the dehydrogenase and esterase reactions was consistent with this two-site model.

The fact that p-nitrophenyl acetate bound to the proposed aldehyde site (cysteine 49) could mean that ester was actually hydrolyzed at that site as well as at the esterase site (cysteine 162). Although aldehyde binds to the esterase site, it may not be oxidized if the orientation between the nicotinamide ring of NAD and the hydride to be transferred is not correct. The possibility that the dehydrogenase site could hydrolyze esterase offers an explanation for the observation that a low percent of the esters could be reduced to aldehydes (5, 9, 10) and justifies the conclusion reached by others that a common active site existed (11).

Although it may not be possible at this time to delineate the fine points of the overall catalytic process, the combined data in this paper and in the accompanying paper (15) show that the enzyme has two separate active sites per subunit. This is in agreement to what was proposed by Blackwell et al. (7) and supported by Kitson (25). In the absence of Mg²⁺ ions it was difficult to find kinetic evidence for the existence of separate active sites. This may be the reason why other investigators have reached the conclusion that only one site exists. One of those sites, most likely containing cysteine 49, was responsible for the dehydrogenase activity of the enzyme. The other, containing cysteine 162 could represent the separate esterase site.

A further component of the active site might be histidine. Previous work revealed that an amino acid with a pKᵥ of 7.2 was involved in the catalytic process (26). In the accompanying paper (15) it was suggested that the catalytic domain is located in the N-terminal portion of the enzyme. Three histidines were found at positions 23, 83, and 140 in human mitochondrial aldehyde dehydrogenase while the remaining two were at positions 235 and 291 (16). It is of interest to note that in human liver, cytosolic aldehyde dehydrogenase
histidine was found at position 29 but not at 83 or 140 (27). Thus, histidine at position 29 is a good candidate for a group functioning as a general base in the deacylation reaction. Now that there is some indication as to the residues at the active site of aldehyde dehydrogenase it is possible to design site-specific mutation experiments to verify these conclusions.

Finding two separate active sites in an enzyme is not uncommon (28, 29). It is difficult to present arguments as to why aldehyde dehydrogenase might have this second site. It is possible that the second site simply represents a region on the enzyme surface where an activated hydrophobic ester can bind. Hence, the region at cysteine 162 may not truly be an "active site" but simply be a reactive cysteine capable of hydrolyzing activated esters such as nitrophenyl acetate. Aldehyde dehydrogenase, although, did not reveal any sequence homology between the esterases or proteases. It was noted, however, that residues 160–163 were very similar to those found in serine protease (15). The physiological significance, if any, of this proposed second site is not obvious.

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Addendum—It was recently reported that glutamate 268 could be a component of the active site of aldehyde dehydrogenase (30).

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