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ENZYMATIC REQUIREMENT FOR CYANAMIDE INACTIVATION OF RAT LIVER ALDEHYDE DEHYDROGENASE*

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Abstract—The *in vitro* inactivation of aldehyde dehydrogenase (ALDH) by cyanamide in rat liver slices, in intact mitochondria, and at various stages of purity was characterized. Low-K_m ALDH was more susceptible to cyanamide inactivation than was the high-K_m form. In addition, the presence of NAD or NADH was necessary for cyanamide inhibition of the ALDH activity. Cyanamide at low concentrations required enzymatic conversion to a reactive derivative that could inhibit ALDH. The data in this study are consistent with the suggestion of DeMaster *et al.* [*Biochem. biophys. Res. Commun.*, **122**, 358 (1984)] that catalase is the cyanamide-converting enzyme. An inhibitor of catalase activity, malonate, decreased the rate of cyanamide inactivation of ALDH in intact mitochondria. Furthermore, affinity chromatography-purified ALDH, free of catalase activity, was not susceptible to cyanamide inactivation. This affinity-purified ALDH was only inactivated by high concentrations of cyanamide. Thus, an alternative pathway for ALDH inactivation may exist in which enzymatic modification of cyanamide is not necessary. It is more likely, however, that a contaminating enzyme in the ALDH preparation is capable of activating cyanamide.

Aldehyde dehydrogenase (aldehyde:NAD oxidoreductase, EC 1.2.1.3, ALDH‡) converts acetaldehyde to acetate, the second step in ethanol metabolism. It is a ubiquitous enzyme found in most organs of the body and in most subcellular organelles [1, 2]. ALDH can be loosely grouped in two categories based on its affinity for acetaldehyde: low-K_m ALDH with a K_m of about 1 μM and high-K_m ALDH with K_m values of 1 mM or greater. Although the exact physiological role of all forms of the enzyme is uncertain, ALDH is involved in catecholamine metabolism [3–5] and in detoxication reactions [2, 6–8].

Inhibition of ALDH is a common element of the treatment of alcoholism [9]. Although many drugs inhibit ALDH [10, 11], two, disulfiram (Antabuse) and citrated calcium carbimide (calcium cyanamide, Temposil), are often prescribed.

Little is known about the mechanism of inhibition of ALDH activity by calcium carbimide. The residues modified on ALDH have not been identified. The drug, which is insoluble in water, must be hydrolyzed by acid in the stomach to a water-soluble compound, cyanamide, prior to absorption by the body [12, 13]. It has also been reported that cyanamide is converted to its *N*-acetyl derivative prior to excretion [14]. Calcium carbimide has been shown to be more specific than disulfiram with respect to inhibition of ALDH isozymes. It inhibits high-K_m ALDH to a much lesser degree than it inhibits low-K_m ALDH [15]. In addition, low-K_m ALDH in livers actively metabolizing ethanol is protected from cyanamide inhibition, leading to the suggestion that cyanamide interacts with the aldehyde binding site on the enzyme [16].

Cyanamide has been reported to have no effect *in vitro* on ALDH purified from sheep, rabbit, cow or mouse liver [17–19]. DeMaster *et al.* [20], among others, conjectured that conversion of cyanamide to a reactive form is required for inhibition of ALDH activity. Since pure yeast ALDH was inhibited by cyanamide only when rat mitochondria were included in the incubation, it was hypothesized that the mitochondrial preparation had supplied a cyanamide-converting enzyme. Recently, DeMaster *et al.* [21] showed that catalase can convert cyanamide to a form that inhibits yeast ALDH.

Acetaldehyde metabolism in the rat occurs in the mitochondria [22, 23]. Partially-purified low-K_m rat mitochondrial ALDH, unlike ALDH from other species, was sensitive to *in vitro* inactivation by cyanamide in some studies [24, 25] but not in others [15]. The inhibition of ALDH activity may have been caused by contamination by the purported cyanamide-converting enzyme in some of the ALDH preparations.

In the present study, experiments were undertaken to determine whether cyanamide must be modified before it can inhibit rat liver low-K_m ALDH. Specificity of isozyme inhibition and rates of inactivation were also measured.

MATERIALS AND METHODS

Acetaldehyde purchased from Aldrich was used without further purification. Matrex Gel Blue A

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‡ Abbreviations: ALDH, aldehyde dehydrogenase; C, cyanamide; and C*, activated cyanamide.

was from Amicon, and Sephacryl S-300 was from Pharmacia Fine Chemicals. Bovine serum albumin (Fraction V), bovine catalase, cyanamide, Triton X-100, NAD, NADH, Tris, sodium fumarate and ammonium sulfate were purchased from Sigma. EDTA, hydrogen peroxide and potassium phosphate were from Fisher and sodium glutamate was from Matheson. Pyrazole was from Eastman. All other chemicals were purchased from Mallinckrodt or obtained from the Biochemistry Department storeroom.

Mitchondrial isolation. A 300–400 g male Wistar rat from the Purdue University Animal Breeding Facility was killed by cervical dislocation. The liver was quickly removed and placed into ice-cold 5 mM Tris–HCl (pH 7.5) containing 0.25 M sucrose and 1 mM EDTA (isolation buffer). The liver was blotted dry, minced in a cold tissue grinder, and then homogenized as a 30% (w/v) suspension in fresh isolation buffer in a glass tube with a Teflon pestle rotating at 600 rpm for three passes. Four additional volumes of isolation buffer were added, and the mitochondria were isolated by differential centrifugation [26].

Partial purifications of ALDH. Mitochondria were lysed by two 20-sec sonic treatments at 0° using a Branson Sonifier Cell Disruptor set at 5.5 and then subjected to centrifugation at 4° at 40,000 *g* for 1 hr. All subsequent purification steps were also performed at 4°.

Solid ammonium sulfate was added to the sample to 40% saturation, and then the solution was stirred for 1 hr. The pH of the solution was maintained at 7 with 1 M ammonium hydroxide. The precipitated protein was removed by centrifugation at 40,000 *g* for 20 min, and then solid ammonium sulfate was added to the supernatant fraction to 60% saturation. After stirring for an hour, centrifugation was again performed for 20 min at 40,000 *g*. The supernatant fraction was discarded, and the pellet was dissolved in a minimal amount of 10 mM sodium phosphate (pH 7.4) containing 2 mM dithiothreitol and 1 mM EDTA (enzyme buffer) and then dialyzed overnight against enzyme buffer.

In gel filtration using Sephacryl S-300, a 26 × 1 cm column equilibrated with enzyme buffer was used. A 1-ml sample of mitochondrial homogenate was applied, and 0.5-ml fractions were collected. The tubes containing ALDH activity were pooled.

Affinity chromatography using Matrex Gel Blue A, an NAD-analog affinity resin, was also used. Ten milliliters of resin was saturated with bovine serum albumin dissolved in enzyme buffer [27] and washed with five column volumes of enzyme buffer. Mitochondrial homogenate (2 ml) was then applied to the column, followed by washing with two column volumes of enzyme buffer. The enzyme was eluted with 5 mM NAD in enzyme buffer.

Incubations. All incubations were performed at 23–25°. Liver slices (50–100 mg) were prepared using a Stadie Riggs tissue slicer. The slices were incubated in Krebs improved Ringer II buffer saturated with 95% O_2/5% CO_2 [28]. At the end of the incubation, the slices were blotted dry and separately homogenized in 0.5 ml of enzyme buffer using a glass-on-glass hand-held homogenizer. The homogenates were sonicated twice for 15 sec prior to assay of enzyme activities (see below). Incubations using partially-purified ALDH were performed in the presence of 0.5 mM NAD in either 100 mM sodium phosphate buffer (pH 7.4) or mitochondrial incubation buffer (see below).

All mitochondrial incubations were carried out in 10 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 10 mM KCl, 5 mM $MgSO_4$ and 1 mM EDTA (mitochondrial incubation buffer) saturated with 95% O_2/5% CO_2. At the end of the incubation period, 100 μl of the mitochondrial suspension were pipetted into 900 μl of 100 mM sodium

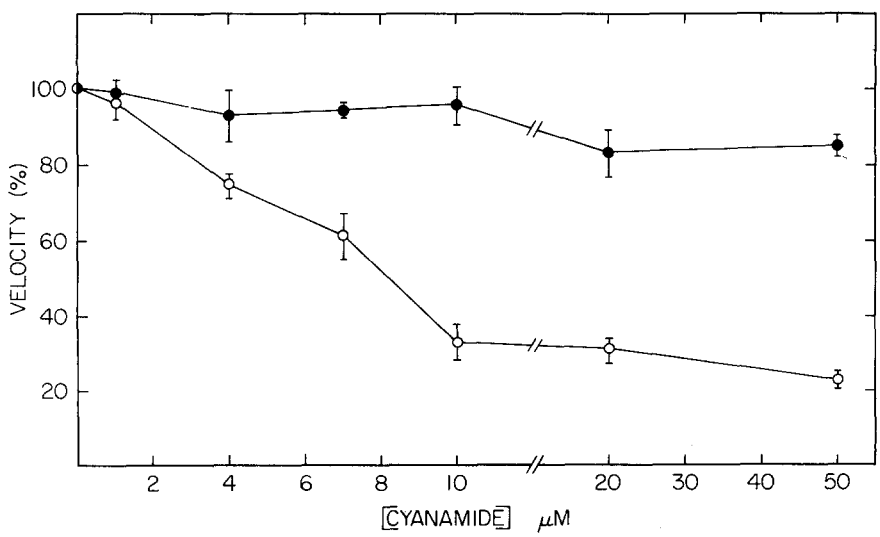

Fig. 1. Inhibition of high- and low-K_m ALDH in rat liver slices. Slices were incubated for 10 min in the presence of various concentrations of cyanamide. At the end of this period, the slices were homogenized and ALDH activity assays of low-K_m (○–○) and high-K_m (●–●) isozymes were performed. Data are presented as means ± S.E. for three animals. Three slices were used from each animal for each concentration.

phosphate buffer (pH 7.4) containing 0.1% (v/v) Triton X-100 and assayed for ALDH activity as described below.

Incubations containing both intact mitochondria and purified ALDH were performed in mitochondrial incubation media. The mitochondria were incubated for 15 min with 2.5 μM cyanamide, affinity-purified ALDH was added, and the incubation was continued from 10 min. The remaining ALDH activity was measured as described for the mitochondrial incubations. Residual ALDH activity from the intact mitochondria was measured in separate experiments by incubating intact mitochondria with 2.5 μM cyanamide for 25 min and assaying the ALDH activity. The residual activity was subtracted from the activity in the assays containing both intact mitochondria and purified ALDH.

Enzyme assays. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out as described in the Hoefer catalog [29]. Protein concentration was measured by the method of Lowry *et al.* [30] with bovine serum albumin as the standard.

Catalase activity was measured by following the decrease in absorbance at 240 nm due to peroxide disappearance [31] in a Gilford spectrophotometer. The reaction was performed at 25° in 50 mM sodium phosphate (pH 7.0) containing 13 mM hydrogen peroxide and the enzyme sample. One catalase unit is the enzyme activity which decomposes 1 μmole of hydrogen peroxide per min.

ALDH activity was measured by following the production of NADH fluorometrically in an Aminco Fluoro-Microphotometer or spectrophotometrically as an increase in absorbance at 340 nm. Low-K_m ALDH activity was assayed at 25° in 100 mM sodium phosphate buffer (pH 7.4) containing 0.5 mM NAD and 10 μM acetaldehyde. At 10 μM acetaldehyde, the high-K_m ALDH activity is only 1% V_{max}, and its contribution to the formation of NADH is not significant. Pyrazole (10 mM) was included in assays of ALDH in slice homogenates.

High-K_m ALDH activity assays employed 10 mM acetaldehyde as the substrate. The K_m values for acetaldehyde in slice homogenates were determined to be 0.7 μM and 1 mM (data not shown). Thus, at 10 mM acetaldehyde, the low-K_m ALDH would be completely saturated and the high-K_m enzyme would be 91% saturated. The high-K_m ALDH activities used in this paper are corrected for low-K_m ALDH contribution and for use of a substrate concentration that gives less than V_{max} for high-K_m ALDH activity.

RESULTS

Time course for the inhibition of ALDH by cyanamide. To establish the length of time required for cyanamide to inhibit ALDH activity, rat liver slices and intact liver mitochondria were separately incubated with 10 μM cyanamide for various periods of time. After 10 min ALDH activity was decreased by 70 and 95% in the slice and mitochondrial incubations respectively. No further inhibition of the low-K_m ALDH activity in the slice incubations was observed at longer time periods. It is possible that some of the ALDH activity remaining in the slices could be that of the cytosolic low-K_m ALDH. Alternatively, cyanamide could have bound to cellular constituents, lowering the effective concentration of the drug. Low-K_m ALDH activity in the slices was totally inactivated by 200 μM cyanamide after a 10-min incubation.

Cyanamide inhibition of high- and low-K_m *ALDH in slices.* Slices were incubated with various concentrations of cyanamide, and the high- and the low-K_m ALDH activities were measured (Fig. 1). The low-K_m ALDH activity was much more susceptible to inhibition by cyanamide than was the high-K_m ALDH activity. After treatment with 50 μM cyanamide, more than 80% of the high-K_m ALDH was still active while only 20% of the low-K_m activity remained.

Table 1. Effect of 185 μM cyanamide in the absence and presence of NAD on low-K_m mitochondrial ALDH purified by Sephacryl S-300 gel filtration*

NAD (0.5 mM)	Cyanamide	Activity (nmoles NADH/min)
Low-K_m ALDH activity		
+	−	22.6 ± 0.8
−	−	25.1 ± 0.4
−	+	16.9 ± 0.5
+	+	<0.1
High-K_m ALDH activity		
+	−	12.5 ± 1.0
+	+	11.2 ± 0.4

* A constant amount of ALDH that had been purified by gel filtration was incubated for 10 min in 100 mM sodium phosphate buffer with the inclusions as noted. At the end of the incubation period, 100 μl of the incubation mixture was diluted into 900 μl of 100 mM sodium phosphate (pH 7.4), and the remaining activity was then measured fluorometrically. Results are presented as remaining activity per ml in the incubation mixture and reported as the means ± S.E. for four experiments.

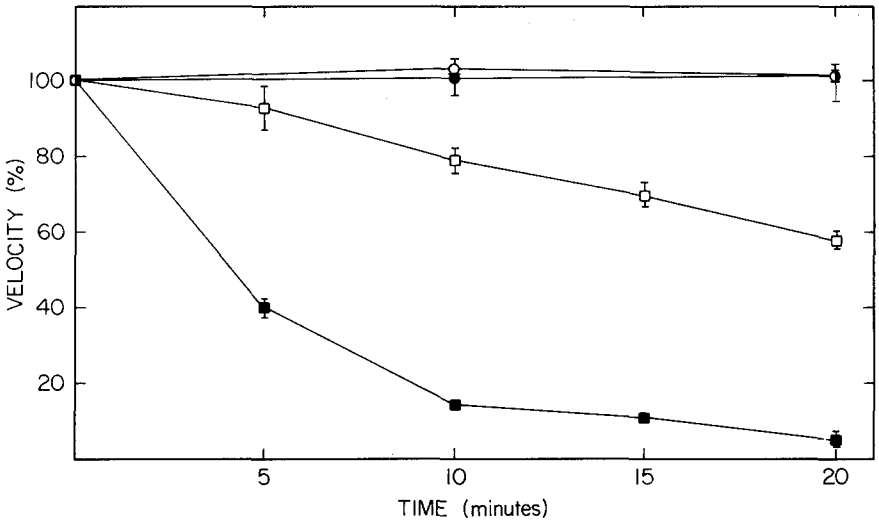

Fig. 2. Protection of low-K_m ALDH in intact mitochondria from cyanamide inhibition by chloral hydrate. Intact mitochondrial incubations and fluorometric assay of ALDH activity were performed as described in Materials and Methods. Incubations were started by the addition of mitochondria. ALDH activity measured immediately after the addition of the mitochondria was designated as 100%. Initial specific activities in the incubations were: no additions (○–○), 6.3; 100 μM chloral hydrate only (●–●), 6.1; 5 μM cyanamide only (■–■), 5.1; and chloral hydrate with cyanamide (□–□), 5.0 nmoles NADH/min per mg protein. Data are presented as the mean ± S.E. for three to five assays.

Coenzyme requirement for ALDH inhibition by cyanamide. Marchner and Tottmar [24] reported that *in vitro* inhibition of rat mitochondrial low-K_m ALDH requires the presence of NAD. To confirm this observation, mitochondrial ALDH was partially purified by ammonium sulfate fractionation. Low-K_m ALDH activity was not inhibited by 4.5 μM cyanamide in a 15-min incubation in the absence of NAD. By contrast, almost 70% inhibition of activity was achieved when 0.5 mM NAD was added to the incubation (15 to 5 nmoles NADH/min). When 70 μM NADH was used in place of NAD, a similar degree of ALDH inhibition was obtained. The low-K_m ALDH was inactivated completely in the presence of NAD by 185 μM cyanamide, the concentration of inhibitor used by Marchner and Tottmar [24].

Mitochondrial ALDH was further purified by Sephacryl S-300 gel filtration. Low-K_m ALDH that had been purified in this manner was also totally inactivated by 185 μM cyanamide in the presence of NAD but not in its absence (Table 1). No inhibition of the high-K_m ALDH activity by cyanamide was observed in the presence of NAD (Table 1).

Protection of ALDH by a substrate analog. Protection experiments (Fig. 2) were performed using chloral hydrate, a known aldehyde-competitive inhibitor [2]. Though the presence of chloral hydrate in the incubation mixture decreased the initial ALDH activity by 17%, in the absence of cyanamide no further change in the low-K_m ALDH activity occurred during the 20-min incubation. Results from control experiments confirmed that cyanamide, in the absence of chloral hydrate, rapidly inactivated low-K_m ALDH. By contrast, when chloral hydrate was included in the incubation, the rate of ALDH inactivation was lowered. The protection of ALDH by chloral hydrate is consistent with, but does not prove, cyanamide binding to the same region on ALDH as does the aldehyde substrate.

Enzymatic activation of cyanamide. Mitochondrial ALDH was purified 25-fold to a specific activity of 140 nmoles NADH/min/mg protein using a Matrex Gel Blue A affinity column. Protein staining after sodium dodecyl sulfate polyacrylamide gel electrophoresis of this preparation revealed only five bands. When the partially purified enzyme was incubated with 2.5 μM cyanamide and NAD, no inhibition of low-K_m ALDH activity was observed (Table 2). To established that this low concentration of cyanamide could have inhibited ALDH activity, intact mitochondria were incubated under the same conditions and a 90% decrease in the ALDH activity was achieved. Incubation of 185 μM cyanamide with the affinity-purified ALDH in the presence of NAD resulted in only 40% inactivation of the enzyme in contrast to 100% inactivation obtained when less pure ALDH was used (Table 1).

Experiments like those of DeMaster *et al.* [20] were also performed to confirm that enzymatic activation of cyanamide is required for rat liver ALDH inhibition. A preparation of intact rat liver mitochondria was added to incubation media containing the affinity-purified ALDH, 2.5 μM cyanamide and NAD. Inclusion of intact mitochondria allowed the cyanamide to significantly inhibit the purified ALDH activity (Table 2). Boiling the mitochondrial preparation abolished its ability to facilitate the cyanamide inactivation of ALDH. These results are consistent with the studies with pure yeast ALDH [20].

Since DeMaster *et al.* have suggested that catalase could be the cyanamide-converting enzyme [21], the various ALDH preparations were assayed for catalase activity. A preparation of washed mitochondria

Table 2. Inhibition of purified ALDH by cyanamide with and without the inclusion of intact mitochondria*

Purified ALDH	Mitochondria	Cyanamide	Activity (nmoles NADH/min)
+	−	−	4.9 ± 0.3
+	−	+	4.7 ± 0.2
+	+	+	1.1 ± 0.1
+	Boiled	+	4.4 ± 0.1

* Affinity-purified enzyme was incubated in the presence of 0.5 mM NAD with additions as noted, and low-K_m ALDH activity assays were performed. Mitochondria were boiled for the one set of experiments for 5 min prior to the incubation. Results are reported as in Table 1 and are the means ± S.E. for three experiments.

isolated by differential centrifugation contained approximately 30% of the catalase present in the original liver homogenate. Assay of an ALDH preparation that had been subjected to Sephacryl S-300 gel filtration revealed that 20% (1600 units/mg protein) of the catalase activity that had been present in the mitochondrial homogenate remained after separation. There was no detectable catalase activity in the affinity-purified ALDH. Addition of 1 mg bovine catalase to affinity-purified ALDH in an incubation mixture containing 5 μm cyanamide and NAD resulted in complete inhibition of low-K_m ALDH activity.

During experiments on a related project, it was noted that malonate decreased the ability of cyanamide to inhibit ALDH activity in intact mitochondria. Whereas in the absence of malonate nearly 100% inhibition of low-K_m activity was obtained, in the presence of 100 mM malonate only a 50% inhibition occurred after a 10-min incubation. This concentration of malonate was found to inhibit catalase, causing a 4-fold decrease in specific activity in a mitochondrial homogenate, from 2600 units/mg mitochondrial protein to 620 units/mg.

Kinetics of cyanamide inactivation of low-K_m *ALDH.* Intact mitochondria in the absence of malonate were incubated with various concentrations of cyanamide for different periods of time. Similar incubations were also performed with affinity-purified ALDH in the presence of NAD. Pseudo first-order graphs of the log of the fraction of remaining activity versus time were constructed, yielding straight lines. Plotting the apparent rate constants, k_{obs}, versus cyanamide concentration ([C]) revealed that the rate of ALDH inactivation approached a maximum value as the cyanamide concentration increased. This is similar to the results of Marchner and Tottmar [24] who suggested the following model and treatment of the data;

$$E + C \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{:}C \xrightarrow{k_{inac}} E - C \tag{1}$$

If the steady-state assumption is made, the rate of reaction is described by Equation 2:

$$-\left[\frac{d(E)}{dt}\right] = k_{obs}(E) = \frac{k_{inac}(E)(C)}{K_I + (C)} \tag{2}$$

where

$$k_I = \frac{k_2 + k_{inac}}{k_1}. \tag{3}$$

Data in the present study, however, revealed the requirement for enzymatic activation of cyanamide. Including the conversion of cyanamide, C to C^*, which may or may not be in equilibrium, leads to Equation 4:

$$C \underset{k_4}{\overset{k_3}{\rightleftharpoons}} C^* \underset{k_2}{\overset{k_1 E}{\rightleftharpoons}} E{:}C^* \xrightarrow{k_{inac}} E - C^* \tag{4}$$

Assuming that k_{inac} is still the rate-limiting step for ALDH inactivation, the rate of reaction would be described by Equation 5:

$$-\left[\frac{dE}{dt}\right] = k_{obs}(E) = \frac{k_{inac}(E)(C_0)}{K_I' + (C_0)} \tag{5}$$

where K_I' is the apparent affinity constant and C_0 is the initial cyanamide concentration. The relationship between K_I and K_I' will vary, depending on whether or not the conversion of C to C^* is reversible. The possible relationships between K_I and K_I' are listed in Table 3. Regardless of the definition of K_I'. Equation 5 may be rearranged to give:

$$\frac{1}{k_{obs}} = \frac{1}{k_{inac}} + \frac{K_I'}{k_{inac}} \cdot \frac{1}{(C_0)} \tag{6}$$

Table 3. Possible relationships between K_I and K_I'*

Conversion of C to C*	K_I'
None ($C \nrightarrow C^*$)	K_I
Reversible equilibrium ($C \rightleftharpoons C^*$)	$\frac{K_I}{1 + K_{eq}}$
Irreversible ($C \rightarrow C^*$)	$\frac{K_I}{1 + e^{-ki}}$

* Definition of terms: K_I is the affinity constant for the two-step mechanism shown in Equation 1; K_I' is the apparent affinity constant based on the mechanism in Equation 4; K_{eq} is the equilibrium constant between C and C^*; k is the rate constant (k_3) for the formation of C^* from C.

Table 4. Kinetic constants for *in vitro* cyanamide inhibition of low-K_m rat mitochondrial ALDH

Enzyme source	Apparent K_I (μM)	k_{inac} (sec × 1000)
Marchner and Tottmar [24]	3	3.6
Intact mitochondria		
− Malonate	17	22
+ Malonate (0.1 M)	250	17
Purified ALDH*	1200	8

* ALDH was purified by affinity chromatography and incubated in the presence of 0.5 mM NAD.

Graphs of $1/k_{obs}$ versus 1/[Cyanamide] were constructed (Fig. 3) and were used to calculate k_{inac} and K_I' (Table 4).

DISCUSSION

Aldehyde dehydrogenase was subjected to partial purification prior to studying its inactivation by cyanamide for two reasons. First, so the role of cofactors such as NAD and substrate could be evaluated in the inactivation process. The second reason was to verify the existence of a cyanamide-activating enzyme. Fractionation of crude mitochondrial homogenates with ammonium sulfate or passage of the homogenate through a gel filtration column removed small molecules from the preparation. The efficacy of cyanamide as an inactivator of ALDH was also abolished. Addition of NAD or NADH at a concentration approximately half that found in mitochondria [32] to the incubation medium restored the ability of cyanamide to inhibit low-K_m ALDH. The requirement for coenzyme is in agreement with a previous study [24]. Inasmuch as the inner mitochondrial membrane is relatively impermeable to NAD [33], no added cofactor was needed to achieve ALDH inhibition in experiments with the intact mitochondria.

An explanation for the requirement for NAD in the inactivation process can be made. Mammalian ALDH, like many other dehydrogenases [34], exhibits ordered sequential binding of substrates, binding first NAD and then aldehyde [35]. Takahashi and Weiner [36] found that the binding of NAD to the enzyme increases the nucleophilicity of the active site. If cyanamide were reacting in the substrate binding site, it can be expected that ordered binding for it would occur. Thus, the analogy between the ordered binding of substrates and the requirement for NAD for ALDH inactivation by cyanamide [24], confirmed in this study, is evidence for cyanamide binding to the same form of the enzyme as does the aldehyde.

Additional evidence exists to support the notion that cyanamide reacts with the aldehyde site. ALDH in livers actively metabolizing ethanol is not inactivated by cyanamide [16]. This was interpreted as implying that acetaldehyde afforded protection for the enzyme. In the present study it was observed that chloral hydrate, a known aldehyde-competitive inhibitor, protected ALDH from cyanamide inactivation.

Having established the requirement for NAD, a cyanamide-activating enzyme was sought. Inhibition of yeast ALDH by cyanamide has been shown to definitely require enzymatic activation of the inhibitor [20]. During the course of this investigation, DeMaster *et al.* [21] reported that catalase is able to catalyze this conversion in an *in vitro* system and also is involved *in vivo*. We therefore examined our ALDH preparations for catalase activity. Assay of a lysed mitochondrial preparation revealed that more than one-fourth of the catalase that had been present in the liver homogenate remained after centrifugation and washing. This was surprising since cata-

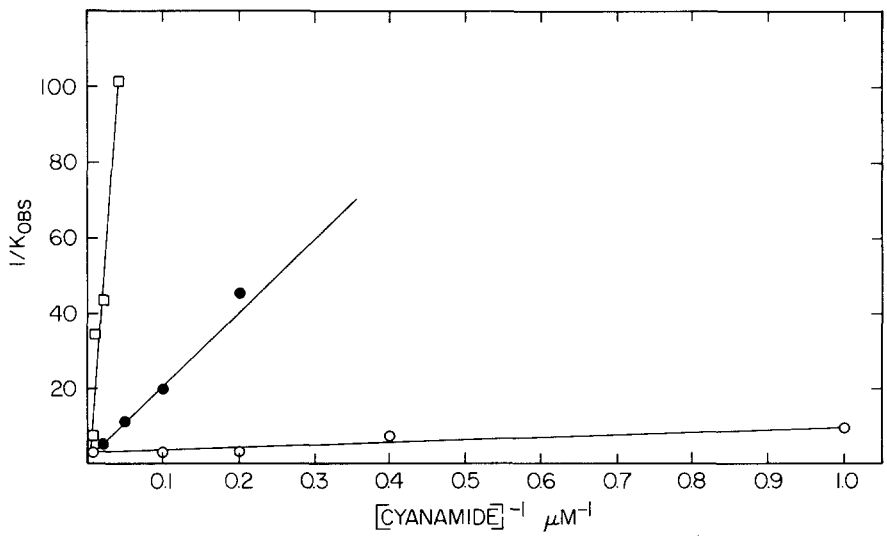

Fig. 3. Kinetics of low-K_m ALDH inhibition by cyanamide. Data are from incubations with intact mitochondria alone (○–○), intact mitochondria in the presence of malonate (●–●) and for affinity-purified enzyme in the presence of 0.5 mM NAD (□–□). Double-reciprocal graphs of pseudo first-order rate constants ($1/k_{obs}$) vs cyanamide concentration ($1/[C]$) were used to determine kinetic values.

lase is a marker enzyme for peroxisomes and is not usually found in mitochondria [37, 38]. The density of peroxisomes is very close to that of the mitochondria (1.23 versus 1.18 g/cm^3 respectively) [39], and so the two organelles are not completely separated by differential centrifugation as performed in this study.

Low concentrations of cyanamide were able to inhibit ALDH activity after the enzyme mixture had been fractionated on the basis of molecular weight. Therefore, the cyanamide-converting enzyme must be about the same size as ALDH. The molecular weight of rat mitochondrial low-K_m ALDH has been reported to be 320,000 [40]. This is much higher than that of mitochondrial ALDH from other species which are in the range of 210,000–260,000 [41, 42]. Thus catalase, with a molecular weight of approximately 250,000 [38], could co-elute with ALDH during gel filtration. Approximately 20% of the catalase activity in the original homogenate was found to be present after fractionation. The relatively large catalase contamination of the ALDH indicates that the molecular weight of mitochondrial ALDH from Wistar rats is more consistent with that reported for ALDH from other animal livers.

A further indication that catalase was the converting enzyme was the decreased inhibition of ALDH by cyanamide in intact mitochondria found in the presence of malonate. It was found that the concentration of malonate used in the incubations inhibited catalase activity by 75%. Since malonate has been shown to bind metal ions [43], the inhibition of catalase activity may be due to chelation of the iron center of the enzyme. Malonate, an inhibitor of the tricarboxylic acid cycle, could have changed the sensitivity of the ALDH to cyanamide inactivation through alteration of the NAD/NADH ratio in the mitochondria. However, since either NADH or NAD can facilitate cyanamide inhibition of ALDH activity, the effect of malonate is probably directly due to its action on catalase. During preparation of this manuscript other investigators reported that a more traditional inhibitor of catalse, 3-aminotriazole, also prevents cyanamide from inactivating low-K_m ALDH [21].

Catalase was separated from ALDH by NAD-analog affinity chromatography. Low concentrations of cyanamide no longer caused inhibition of low-K_m ALDH activity. The addition of catalase to this affinity-purified ALDH. however, restored the ability of cyanamide to inactivate ALDH. Thus, the data in this study confirms the role of catalase as the cyanamide-converting enzyme at low cyanamide concentrations.

Since cyanamide underwent enzymatic modification, additional steps were required for the previously-proposed mechanism for ALDH inactivation (Equation 4). The rate at which the noncovalent E:C* complex is converted to the covalent E – C* adduct, k_{inac}, is presumed to be the rate-limiting step of ALDH inactivation since saturation was observed (Fig. 3). Double-reciprocal graphs of the pseudo first-order rate constants versus cyanamide concentration allowed for the calculation of the kinetic constants K_I' and k_{inac} (Equation 6).

The value for k_{inac} was approximately equal in all three systems used in this study and is relatively close to that reported previously [24]. This equivalence could be fortuitous or could indicate that the same E:C* complex is involved in the rate-limiting step in each case.

The affinity term, K_I, in its most simple form contains only the dissociation constant of the E:C* complex and the rate of inactivation (Equation 3). In these experiments, however, the apparent K_I term, K_I', was also affected by the rate at which cyanamide is converted to the reactive form (Table 3). Since the k_{inac} terms for the different systems are similar, K_I' should be useful in comparison of the availability of activated inhibitor for binding with ALDH. The results of the malonate experiments are consistent with this notion. Inclusion of malonate in the intact mitochondrial incubations caused a 75% inhibition of catalase. The K_I' increased, indicating a decrease in the affinity of ALDH for the inhibitor (Table 4).

Cyanamide inhibition of affinity-purified ALDH is harder to rationalize. Since no catalase activity was measurable in the purified enzyme preparation, ALDH should have been resistant to cyanamide, independent of cyanamide concentration. At high concentrations of inhibitor, however, ALDH inactivation did occur. It is possible that cyanamide exists in equilibrium with an activated form. Only at high concentrations would enough of the activated form be present to inhibit ALDH activity. It is unlikely though that this form of cyanamide would be the same as that produced by an oxidizing enzyme such as catalase. An activated form of cyanamide need not exist since cyanamide itself could be attacked slowly by the nucleophilic amino acid.

Yeast ALDH has been reported to be totally resistant to inhibition by cyanamide [20]. Highly purified horse liver ALDH available in this laboratory was also found not to be inactivated by high concentrations of cyanamide. Thus, it is possible that a contaminating enzyme present in the affinity-purified rat ALDH preparation converts cyanamide to an activated form.

Now that a cyanamide-activating enzyme has been found, experiments can be performed to isolate and characterize the activated form of cyanamide. In addition, studies with purified ALDH that has been inactivated by cyanamide in the presence of catalase can be performed to study the stoichiometry and chemical interactions that occur.

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