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INACTIVATION OF LOW-K_m RAT LIVER MITOCHONDRIAL ALDEHYDE DEHYDROGENASE BY CYANAMIDE *IN VITRO*

A CATALASE-MEDIATED REACTION*

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Abstract—The inactivation of the affinity chromatography purified low-K_m rat liver mitochondrial aldehyde dehydrogenase (ALDH)—free of catalase activity—by the alcohol sensitizing agent cyanamide was studied *in vitro*. This ALDH-purified preparation was not susceptible to cyanamide inactivation at concentrations up to 2.5 mM. On the other hand, ALDH activity appears to be irreversibly inhibited when the incubation mixture contained ALDH, catalase, NAD^+ and cyanamide. Influence of catalase, NAD^+ and cyanamide concentrations in the incubation mixtures on the ALDH activity were also established. The time course of the concentration of cyanamide in an incubation mixture when ALDH activity was inhibited by cyanamide in the presence of catalase and NAD^+, was evaluated by HPLC. No disappearance of cyanamide was observed for a period of time up to 24 hr. This result suggests that no metabolic conversion of cyanamide to an active inhibitory form takes place, as has been suggested recently.

Calcium carbimide in the citrated form (Temposil®, Abstem®, Dipsan®) or as an aqueous cyanamide solution (Colme®) is commonly referred to as an antialcohol, an alcohol deterrent or an alcohol-sensitizing drug, due to detrimental effects produced during its interaction with ethanol [1]. The alcohol-sensitizing effect is mainly due to the inactivation of aldehyde dehydrogenase (aldehyde: NAD^+ oxidoreductase, EC 1.2.1.3 ALDH‡), which causes an elevated blood acetaldehyde level in animals, as well as man, after ethanol ingestion [2–8], and results in a number of undesirable effects such as tachycardia, hypotension, flushing and dypsnea, thereby deterring further drinking.

It has been described that cyanamide is a potent inhibitor of rat liver ALDH isozymes *in vivo* [2–4, 7, 9–12] in isolated hepatocytes [13], isolated mitochondria [14] or liver slices [15]. In contrast, cyanamide does not inhibit *in vitro* the purified liver ALDH from mouse [10], rabbit and cow [9] or sheep [16]. However, the inactivation *in vitro* of ALDH has been observed using partially-purified enzyme preparations from sheep [17] and rat [11] or with pure yeast ALDH in the presence of either rat intact mitochondria and isolated microsomes [18, 19]. These results led some authors [9, 10, 16, 18, 20] to hypothesize an enzyme catalysed conversion of cyanamide to an active metabolite, which would be responsible for ALDH inactivation.

The major metabolic pathway for cyanamide in rat, rabbit, dog and man involves acetylation catalysed by an acetyl-*S*-CoA dependent hepatic *N*-acetyltransferase yielding a product which is excreted in the urine [21]. Since the reaction product, *N*-acetylcyanamide, did not inhibit ALDH *in vitro* even in the presence of the cyanamide-activating enzyme, a second—albeit minor—pathway for cyanamide metabolism was proposed [15, 19, 22]. Recently, DeMaster *et al.* [20] have shown that catalase is the enzyme responsible for the metabolic cyanamide activation. Many efforts have been directed to identify the products of this catalase-mediated reaction and recently, Shirota *et al.* have detected cyanide as a product of cyanamide transformation *in vitro* catalysed by bovine liver catalase [23] or by rat liver microsomes [24].

Although there are some kinetic studies about low-K_m rat liver mitochondrial ALDH inactivation by cyanamide *in vitro* [15, 25], the necessary detailed requirements and conditions to produce the inactivation on purified rat ALDH free of catalase are not described. In this paper, a purification method to obtain the low-K_m rat liver mitochondrial ALDH free of catalase was used to characterize the *in vitro* inactivation. Experiments were undertaken to determine whether cyanamide concentration was modified in long-term incubations where ALDH has been inactivated by cyanamide in the presence of NAD^+ and catalase.

MATERIALS AND METHODS

Chemicals. Acetaldehyde purchased from Merck (Darmstadt, F.R.G.) was used without further purification. Acetonitrile (HPLC grade) was from Farmitalia Carlo Erba (Milan, Italy). Bovine serum albumin (fraction V) and bovine liver catalase were

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‡ Abbreviations used: ALDH, aldehyde dehydrogenase.

purchased from Sigma Chemical Co. (St Louis, MO). NAD^+ was supplied by Boehringer Männheim (Männheim, F.R.G.) and cyanamide by Fluka (Buchs, Switzerland). Sephadex G-25 (prepacked PD10 columns) and 5′-AMP-Sepharose 4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Dansyl chloride (for fluorescence labelling of amino acids) and all other chemicals, analytical grade, were purchased from Merck.

Enzyme preparation. The primary aim of the enzyme purification was to obtain a preparation of low-K_m rat liver mitochondrial ALDH free from catalase and the other form of the mitochondrial enzyme with a high-K_m value for acetaldehyde. All steps were carried out at 0–4°. Male Sprague–Dawley rats (240–300 g) from Charles River (France), fasted overnight, were killed by stunning and subsequent decapitation. The livers were perfused *in situ* with cold 0.25 M sucrose solution, cut into small pieces and 30 g of tissue homogenized in a Potter–Elvejhem homogenizer with 0.25 M sucrose–0.1% 2-mercaptoethanol solution. The homogenate was made up to 10% (w/v) and centrifuged as described by Tottmar *et al.* [26] to obtain the mitochondrial fraction. The pellet (mitochondrial fraction) was gently suspended in 0.25 M sucrose–0.1% 2-mercaptoethanol solution (5 ml/g of liver) and washed two times to reduce the content of cytosolic enzymes. The final pellet was suspended in 60 ml (2 ml/g of liver) of 10 mM Na^+-phosphate buffer (pH 7.5) and sonicated for 6 × 15 sec using a Labsonic 2000 Disintegrator provided with a 127 × 9.5 mm titanium probe set at 50 Ω.

The sonicated suspension was dialysed overnight against 30 mM Na^+-phosphate buffer (pH 6.0) containing 1 mM EDTA and 0.1% 2-mercaptoethanol (buffer 1), and then centrifugated at 87,000 *g* for 30 min. The clear supernatant (mitochondrial extract) was filtered through a Millipore filter of 0.45 μm pore size. The mitochondrial extract (13.6 and 3.3 units of low and high-K_m ALDH, respectively) was chromatographed through a 5′-AMP-Sepharose column (1.6 × 6.5 cm) equilibrated with buffer 1. The column was washed with buffer 1 (seven column volumes) until unretained protein was eluted (catalase eluted at this stage). The low-K_m ALDH was eluted with approximately five column volumes of 30 mM Na^+-phosphate buffer (pH 8.0) containing 1 mM EDTA and 0.1% 2-mercaptoethanol (buffer 2). Finally, the high-K_m ALDH was eluted with three column volumes of buffer 2 containing 0.5 mM NAD^+. The fractions containing low-K_m ALDH were pooled, adjusted to pH 7.0 by Na^+-phosphate acid addition and made 20% in glycerol. Aliquots of this purified preparation were stored at −22° and no loss of activity was observed for at least 12 months. The specific activity of the enzyme preparation was 342 nmol of NADH formed per min per mg of protein (measured with 50 μM acetaldehyde, 1 mM NAD^+ in 50 mM Na^+-pyrophosphate, pH 9.3, at 25°), which is a six-fold purification with respect to the specific activity of the crude mitochondrial extract. Before inactivation experiments, low-K_m ALDH preparation was thawed and filtered through a PD10 column equilibrated with incubation media buffer to obtain a solution of 0.8 mg protein/ml. This solution must be maintained in an ice-water bath for 2 hr prior to experimentation in order to attain a complete reactivation of the enzyme.

Protein was determined by the method of Bensandoun and Weinstein [27] with bovine serum albumin as the standard.

Incubations. For the inactivation experiments, incubations were carried out at 30 ± 0.1°, in 25 mM Na^+-phosphate buffer (pH 7.4) containing 0.25 M sucrose, 10 mM KCl, 5 mM $MgSO_4$ and 1 mM EDTA (incubation media buffer) if not otherwise stated.

Enzyme assays and kinetic studies. ALDH activity was measured by following the production of NADH spectrophotometrically at 340 nm in a Shimadzu CL-720 microanalyser spectrophotometer. Low-K_m ALDH activity was assayed at 25 ± 0.1° in 50 mM Na^+-pyrophosphate buffer (pH 9.3) containing 1 mM NAD^+ and 50 μM acetaldehyde. Total ALDH activity employed 5 mM acetaldehyde as the substrate and high-K_m ALDH activity was determined by difference between total and low-K_m ALDH activities. One ALDH unit is the enzyme activity which produces 1 μmol of NADH per min in standard conditions.

For the inactivation experiments, ALDH preparation (80 μg of protein) was incubated with NAD^+, catalase and cyanamide in incubation media buffer. The inactivation was initiated by the addition of cyanamide if not otherwise stated. At various or fixed times, aliquots were withdrawn and transferred to the spectrophotometer cuvette. The spectrophotometric reaction was initiated by the addition of acetaldehyde.

Catalase activity was determined as described by Aebi [28].

Pseudo-first-order rate constants were calculated by a nonlinear regression program using the least squares method [29]. Lines were fitted to the experimental points by the method of least squares.

Determination of the cyanamide concentration in incubates. The cyanamide concentration was evaluated by the method described by Pruñonosa *et al.* [30] with some modifications in the sample preparation.

Aliquots of 0.1 ml from incubates, previously diluted 1:1 in incubation media buffer where sucrose was omitted, were withdrawn at desired times and transferred to tubes containing 0.5 ml of 2 μM α-phenylglycine in methanol solution (internal standard). This solution was diluted to 0.2 mM in 0.1 N sodium hydroxide and then made 2 μM by dilution in methanol, which served as a deproteinization agent. The tubes were shaken and their content filtered through a Millipore filter of 0.45 μm pore size. Aliquots of 0.5 ml of filtrates were transferred to silanized conical glass reacti-vials and evaporated to dryness with a stream of nitrogen at 40°. The residues were redissolved in 100 μl of 0.2 M sodium carbonate-sodium bicarbonate (pH 9.0) buffer solution and derivatized with dansyl chloride as described earlier [30]. Standards containing known amounts of cyanamide were processed in parallel.

RESULTS

Catalase and NAD^+ or NADH requirement for ALDH inactivation by cyanamide in vitro

As shown in Table 1, the *in vitro* inactivation of

Table 1. Effect of NAD(H) and catalase in the inactivation of low-K_m mitochondrial ALDH by cyanamide.

(A)	(B)	(C)	(D)	Activity		P value vs paired control
				10^2 units/ml	% of control	
−	−	−	−	4.13 ± 0.036	100	—
−	−	−	+	4.21 ± 0.044	101.9 ± 1.4	N.S.
+	−	−	−	4.68 ± 0.086	100	—
+	−	−	+	4.72 ± 0.079	100.8 ± 2.5	N.S.
−	−	+	−	4.21 ± 0.061	100	—
−	−	+	+	4.20 ± 0.049	99.8 ± 1.8	N.S.
+	−	+	−	4.28 ± 0.064	100	—
+	−	+	+	2.90 ± 0.058	67.8 ± 1.7	<0.001
−	+	−	−	4.60 ± 0.023	100	—
−	+	−	+	4.48 ± 0.038	97.4 ± 0.96	N.S.
−	+	+	−	4.44 ± 0.040	100	—
−	+	+	+	1.55 ± 0.023	34.9 ± 0.61	<0.001

(A) NAD = 0.5 mM; (B) NADH = 0.5 mM; (C) Catalase = 750 U/ml; (D) Cyanamide = 200 μM.

A constant amount of low-K_m ALDH was incubated for 10 min in incubation media buffer (0.5 ml final volume) with the inclusions as noted. At the end of the incubation period, 50 μl of the incubation mixtures were taken to determine ALDH activity in a 1.25 ml final volume cuvette. All values are means ± SE for three assays. Statistical comparisons are based on Student's *t*-test.

low-K_m rat liver mitochondrial ALDH by cyanamide required the co-incubation of the enzyme with NAD^+ or NADH, catalase and the inhibitor. Different sensitivity was observed using NAD^+ or NADH. The coenzyme requirement agrees with the results reported by Marchner and Tottmar [25] for NAD^+ (saturating concentrations) or Svanas and Weiner [15] for NAD^+ (saturating concentrations) and NADH. Catalase requirement is in accord with previous findings as described by DeMaster *et al.* [20].

Attempts to generate a possible active form of cyanamide were made by incubation of NAD^+, catalase and cyanamide for 30 min under the conditions described in Table 1. Later addition of low-K_m ALDH did not result in its inhibition when activity was determined immediately; 40% inhibition was achieved when activity was determined after 10-min incubation. When 10 mM sodium azide (a catalase inhibitor) was added prior to low-K_m ALDH addition, the latter enzyme was protected even after 10 min of incubation. These results suggest that no active form of cyanamide had been generated as a result of exposure to catalase.

Svanas and Weiner [15] reported no inhibition of low-K_m ALDH activity (mitochondrial ALDH partially purified by ammonium sulphate fractionation) by 4.5 μM cyanamide in a 15-min incubation in the absence of NAD^+ and almost 70% inhibition of activity when 0.5 mM NAD^+ or 70 μM NADH, used in place of NAD^+, were added to the incubation. However, no explanation for the difference in coenzyme concentrations was given.

We studied in some detail the effect of the concentration of NAD^+ or NADH on the inactivation of low-K_m mitochondrial ALDH by cyanamide. As shown in Fig. 1 both curves of inhibition pass through maximal values. There seems to be an optimum concentration of NAD^+ or NADH around 50 μM for the enzyme inactivation. At this point, little difference in sensitivity to the inactivation can be observed between NAD^+ or NADH.

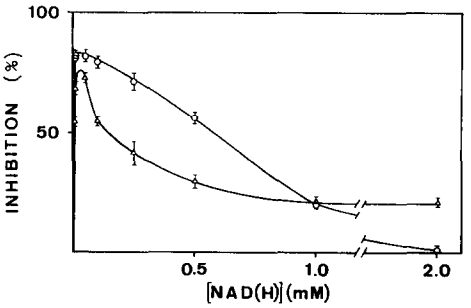

Fig. 1. Effect of the concentration of NAD(H) in the inactivation of low-K_m ALDH. Different samples containing 3.90×10^{-2} U/ml of low-K_m ALDH, 750 U/ml of catalase and 200 μM cyanamide were incubated for 10 min in the presence of various concentrations of NAD^+ (△) and NADH (○). Activities at the end of this period were determined and compared with a paralleled sample which did not contain the coenzyme. Data are presented as the mean ± SE for three assays.

Effect of catalase and cyanamide concentrations in the ALDH inactivation in vitro.

The effect of catalase and cyanamide concentrations in the incubation of low-K_m ALDH *in vitro* were studied in the presence of the optimum concentration of NAD^+. The results obtained for catalase and cyanamide dependences (Fig. 2) showed saturation.

Irreversible inactivation of low-K_m *ALDH by cyanamide* in vitro. Cyanamide has been reported to be an uncompetitive inhibitor of beef ALDH, with respect to both acetaldehyde and NAD^+ [17]. In

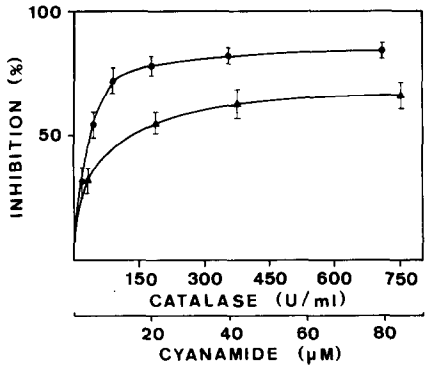

Fig. 2. Effect of the concentrations of catalase and cyanamide on the inactivation of low-K_m ALDH. (▲) Different samples containing 6.51×10^{-2} U/ml of ALDH, 50 μM NAD^+ and 20 μM cyanamide were incubated for 10 min in the presence of various concentrations of catalase. (●) Different samples containing 5.28×10^{-2} U/ml of ALDH, 50 μM NAD^+ and 750 U/ml of catalase were incubated for 10 min in the presence of varying concentrations of cyanamide. Activities at the end of these periods were determined and compared with those of paralleled samples which did not contain cyanamide. Data are presented as the mean ± SE for three assays.

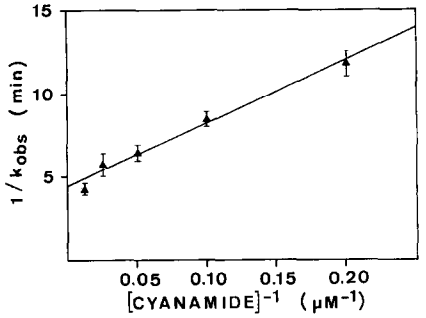

Fig. 3. Double reciprocal plot of the apparent first-order rate constant of low-K_m ALDH inactivation as a function of cyanamide concentration. All incubates contained 5.26×10^{-2} U/ml of ALDH. 50 μM NAD^+ and 750 U/ml of catalase in incubation media buffer.

contrast, it has been reported that a pseudo-irreversible inhibition of low-K_m rat ALDH occurs *in vitro* [25]. We studied the degree of inhibition incubating different amounts of low-K_m ALDH (0.014 to 0.095 U/ml) for 10 min in incubation media buffer in the presence of 50 μM NAD^+ and 750 U/ml of catalase at varying concentrations of cyanamide (0 to 20 μM). At the end of the incubation period, aliquots of the incubation mixtures were withdrawn to determine the remaining ALDH activity. Straight lines were obtained intersecting the abscissa at different points far from the origin, indicating irreversible inhibition. This was confirmed by the lack of restoration of activity by dialysis or by the presence of 5 mM acetaladhyde.

Kinetics of cyanamide inactivation of low-K_m *ALDH.*

The kinetic constants for *in vitro* cyanamide inactivation of affinity-purified low-K_m rat mitochondrial ALDH were obtained according to Marchner and Tottmar [25]. Constant amounts of low-K_m ALDH were incubated with various concentrations of cyanamide for different periods of time in the presence of 50 μM NAD^+ and 900 U/ml of catalase. The inactivations observed were time-dependent in all cases and pseudo-first order graphs of the logarithm of the fraction of remaining activity against time were constructed, yielding straight lines. Pseudo-first order rate constants (k_{obs}) were calculated from the equation:

$$A = A_0 \exp(-k_{obs} t) \quad (1)$$

where A and A_0 represents the activity at t and zero time, respectively. Double reciprocal plot of the apparent first-order rate constant versus inhibitor concentration yielded a straight line (Fig. 3) indicating an approach to a maximal value of the rate of inactivation as the inhibitor concentration was increased. This saturation effect led to Marchner and Tottmar [25] to suggest that reversible complexes are formed between the inhibitor and the enzyme prior to covalent binding, writing the inactivation processes as:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} E{:}I \xrightarrow{k_{+2}} E{-}I \quad (2)$$

where E and $E{:}I$ are the holoenzyme and the reversible holoenzyme-inhibitor complex, respectively, and k_{+2} is the inactivation rate constant for the conversion of $E{:}I$ to the covalent complex $E{-}I$. The rate of enzyme disappearance can be written by the following expression:

$$\frac{-\mathrm{d}(E)}{\mathrm{d}t} = \frac{k_{+2}\,(I)\,(E)}{K_I + (I)} \quad (3)$$

where:

$$k_{obs} = k_{+2}\,(I) / K_I + (I) \quad (4)$$

and

$$K_I = k_{-1} + k_{+2}/k_{+1}. \quad (5)$$

The K_I and k_{+2} values from Eqn (1) in which the k_{obs} was substituted accordingly to Eqn (4) were calculated. Five equations (one for each concentration of inhibitor) were fitted to the experimental points by a nonlinear regression program using the least squares method [29]. The values for K_I and k_{+2} were 10.9 ± 1.89 μM and 208 ± 20.3×10^{-3} min^{-1}, respectively.

Kinetics at different concentrations of catalase gave different K_I values showing a linear relationship between K_I and the reciprocal of catalase concentration whereas k_{+2} remained constant (Table 2).

Effect of acetaldehyde on the inactivation of ALDH

Different concentrations of acetaldehyde were assayed in incubates where low-K_m ALDH had been inactivated by cyanamide. The effect of acetaldehyde was determined by obtaining the apparent first-order

Table 2. Kinetics constants obtained at different concentrations of catalase in the incubation mixtures.

Catalase (U/ml)	Cyanamide (μM)	K_I (μM)	k_{+2} ($10^3 \times min^{-1}$)
900	2.5–5–10–20–40	10.9 ± 1.89	208.1 ± 20.3
450	2.5–5–10–20–40	15.5 ± 2.94	201.4 ± 22.4
225	5–10–20–40–80	17.2 ± 2.04	181.2 ± 11.3
150	5–10–20–40–80	26.3 ± 4.71	200.7 ± 21.1
110	10–20–40–80–160	39.7 ± 6.62	199.5 ± 18.3
60	10–20–40–80–160	77.8 ± 20.08	234.9 ± 36.2
30	40–80–160–320–640	153.8 ± 27.96	188.6 ± 17.4

Constant amounts of low-K_m ALDH were incubated for different periods of time in the presence of 50 μM NAD^+ and various concentrations of cyanamide for each concentration of catalase. The concentrations of cyanamide used in each assay are included in the table.

Table 3. Effect of acetaldehyde on the inactivation of ALDH.

Concentration of acetaldehyde in incubates (μM)	k_{obs} ($10^3 \times min^{-1}$)
0	173.7 ± 15.8
1	124.2 ± 8.6
5	63.4 ± 1.1
50	0

Different samples containing 4.92×10^{-2} U/ml of low-K_m ALDH, 50 μM NADH, 750 U/ml of catalase and 20 μM cyanamide were incubated in the presence of various concentrations of acetaldehyde. Activities were determined at different incubation time for each incubate to obtain k_{obs}.

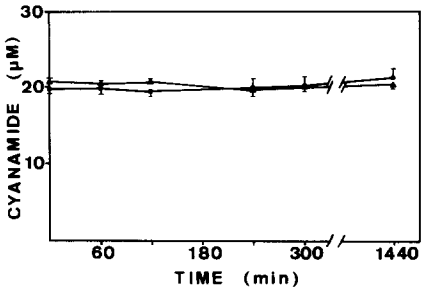

Fig. 4. Time course of the concentration of cyanamide in incubates. Two sets of samples were incubated for 24 hr at room temperature. Incubate A (▲) contained 50 μM NAD^+, 750 U/ml of catalase and 20 μM cyanamide in incubation media buffer. Incubate B (●) contained the same as incubate A and 1.27 U/ml of low-K_m ALDH added. Sucrose from incubation media buffer was omitted in both cases. Each experimental point was determined in triplicate.

rate constants in the presence of different concentrations of acetaldehyde (Table 3). Note the observed decrease in k_{obs} as the concentration of acetaldehyde rises. Concentrations of acetaldehyde of 50 μM in the incubations prevents the inactivation of the enzyme by cyanamide. The results suggest that cyanamide reacts with the aldehyde site of ALDH as suggested by Marchner and Tottmar [11, 25] and Svanas and Weiner [15]. On the other hand, 50 μM ethanol did not prevent this inactivation ($k_{obs\ (without\ ethanol)} = 160.1 \pm 5.64$; $k_{obs\ (50\ \mu M\ ethanol)} = 150.7 \pm 8.08$).

Time course of the concentration of cyanamide during the inactivation of low-K_m ALDH

The concentration of cyanamide as a function of time was evaluated in two sets of incubations. One of them contained NAD^+, catalase and cyanamide in the incubation media buffer (incubate A). The other contained, in addition, low-K_m ALDH (incubate B). In this case, ALDH was being inactivated by cyanamide (no ALDH activity was detected after 30 min of incubation). The aim of this set of incubations was to detect the formation of a possible active form of cyanamide as indicated by the disappearance of the parent compound.

The concentration of cyanamide in the incubates were determined as described in Materials and Methods. The molar ratio of ALDH:cyanamide in the experiment was 1:2 and the results are shown in Fig. 4. No decay in the concentration was observed in either case. These results cast doubt on a catalytic role of catalase in the conversion of cyanamide to an active form as suggested by DeMaster *et al.* [18, 22] and Svanas and Weiner [15].

DISCUSSION

Low-K_m rat liver mitochondrial ALDH was selected in this study for two reasons. First, acetaldehyde metabolism in the rat mainly occurs in the hepatic mitochondria [31, 32]. Second, the low-K_m ALDH is much more sensitive to inactivation by cyanamide than are other ALDHs [33]. Low-K_m ALDH was purified with the aim of achieving an enzymatic preparation free from both catalase and high-K_m mitochondrial ALDH, in order to establish the minimum requirements and conditions to produce the inactivation by cyanamide.

It was shown that low-K_m ALDH inactivation by

cyanamide requires co-incubation of the enzyme, NAD^+ and catalase in the presence of cyanamide (Table 1). This result might suggest the participation of both catalase and NAD^+ in a holoenzyme–inhibitor complex. The catalytic role of catalase in this process is doubtful. No active form of cyanamide was generated when NAD^+, catalase and cyanamide were incubated in the absence of ALDH. The possibility that cyanamide itself is the inhibitor should also be considered. It may be that the inhibitory complex (holoenzyme–inhibitor) consists of ALDH, NAD^+, catalase and cyanamide. The active center of catalase could be related in this binding inasmuch as no inhibitory effect was observed when sodium azide was used to block this center. The cyanamide from the holoenzyme–inhibitor complex could interact with the ALDH center for aldehydes as suggested by the results in Table 3 and result in the irreversible inactivation. The results agree with those obtained in livers actively metabolizing ethanol [11] where ALDH is not inactivated, suggesting that acetaldehyde derived from ethanol metabolism afforded protection for the enzyme. The hypothesis can be correlated with the protective effect against cyanamide inactivation afforded by the aldehyde-competitive inhibitor chloral hydrate [15]. The non-protective effect of ethanol in the inactivation of ALDH by cyanamide *in vitro* pointed out in this paper contrasts with the protective effect described *in vivo* [11]. However, this result could confirm the suggestion that acetaldehyde derived from ethanol metabolism *in vivo* is the responsible of the protective effect for ALDH.

It has been described by Kirkman and Gaetani [34] that catalase has affinity for adenine dinucleotides. Each tetrameric molecule of human or bovine catalase contains four molecules of tightly-bound NADPH and the binding sites have the relative affinities $NADPH > NADH > NADP^+ > NAD^+$ [34]. Thus, the influence of NAD^+ or NADH in the inactivation of low-K_m ALDH (Fig. 1), which did not show a typical saturation pattern, may be explained by the existence of the equilibrium.

$$NAD^+ + \text{catalase} \rightleftarrows NAD^+\text{-catalase}.$$

The NAD^+-catalase complex may compete with free NAD^+ for the coenzyme site of ALDH. The disagreement with the saturation kinetics of NAD^+ reported previously [25] may be explained by the difference in the enzymatic preparations used in both cases.

The relative rate of formation of a reactive cyanamide metabolite catalysed by purified bovine liver catalase was estimated using purified yeast ALDH [22]. These authors used ascorbate, H_2O_2 or a glucose/glucose oxidase system as a source of H_2O_2, to generate the reactive metabolite in the presence of NAD^+, catalase and cyanamide. However, no metabolite was isolated and identified. It is noteworthy that no H_2O_2 or a source of it was used in the present study.

The concentration of cyanamide in an incubate being inactivated the low-K_m ALDH by cyanamide remained constant for up to 24 hr (Fig. 4), supporting the hypothesis that there was no catalytic effect of catalase on cyanamide. These results are also in accord with those obtained by incubating NAD^+, catalase and cyanamide, where no active form of cyanamide was generated. Therefore, the hypothesis that cyanamide itself, in the presence of NAD^+ and catalase, would be the true inhibitor is reinforced.

The affinity constant, K_I, reported in this paper ($K_I = 10.9 \pm 1.89\ \mu M$) is relatively close to that reported previously using a purified low-K_m ALDH-containing catalase ($K_I = 3\ \mu M$) [25] or intact mitochondria ($K_I = 17\ \mu M$) [15]. However, Svanas and Weiner [15] reported a $K_I = 250\ \mu M$ when 0.1 M malonate was added to the intact mitochondrial incubations. Inclusion of malonate caused a 75% inhibition of catalase and the increased K_I in its presence was interpreted as a decrease in the affinity of ALDH for the inhibitor. These results are in accord with the linear relationship observed by us between K_I and the reciprocal of the concentration of catalase (Table 2). Thus, any comparison of K_I values should recognise that K_I varies with the reciprocal of the concentration of catalase used in the incubation mixtures.

The value for k_{+2} reported in this paper is clearly different from those reported previously [15, 25], suggesting that different holoenzyme–inhibitor complexes could be involved in the rate-limiting step in each case. We propose that a holoenzyme–inhibitor complex involving low-K_m ALDH, NAD^+, catalase and cyanamide could constitute an alternative explanation in the mechanism of inactivation of ALDH by cyanamide *in vitro*. Studies to elucidate the true composition of this complex are in progress. Our results point out that inactivation of low-K_m ALDH by cyanamide can be a catalase-mediated reaction, in which catalase would act as a carrier of cyanamide to the active center of ALDH, more than a catalytic effect on cyanamide induced by catalase.

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