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THE METABOLIC ACTIVATION OF CYANAMIDE TO AN INHIBITOR OF ALDEHYDE DEHYDROGENASE IS CATALYZED BY CATALASE

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The inhibition of aldehyde dehydrogenase by cyanamide is dependent on an enzyme catalyzed conversion of the latter to an active metabolite. The following results suggest that catalase is the enzyme responsible for this bioactivation. The elevation of blood acetaldehyde elicited by cyanamide after ethanol administration to rats was attenuated more than 90 percent by pretreatment with the catalase inhibitor, 3-amino-1,2,4-triazole. This attenuation was dose dependent and was accompanied by a reduction in total hepatic catalase activity. Although hepatic catalase was also inhibited by cyanamide, a positive correlation between blood acetaldehyde and hepatic catalase activity was observed. In vitro, the activation of cyanamide was catalyzed by a) the rat liver mitochondrial subcellular fraction, b) the 50-65% ammonium sulfate mitochondrial fraction and c) purified bovine liver catalase. Cyanamide activation was inhibited by sodium azide. Since much of the hepatic catalase is localized in the peroxisomes and since peroxisomes and mitochondria co-sediment, the cyanamide activating enzyme, catalase, is likely of peroxisomal and mitochondrial origin.

The alcohol deterrent agent, cyanamide ($H_2N-C\equiv N$), is a potent in vivo inhibitor of the aldehyde dehydrogenase isozymes (1-4). When cyanamide is administered before ethanol, it causes a pronounced increase in circulating blood acetaldehyde levels in animals as well as man (1,2,5-10). Recently, we demonstrated that the inhibition of aldehyde dehydrogenase by cyanamide was dependent on its conversion to an active form which accounts for the lack of a direct inhibitory effect by cyanamide on purified aldehyde dehydrogenase isozymes (1,5,11,12). Using yeast aldehyde dehydrogenase (EC 1.2.1.5) as a model for the mammalian enzyme, we showed that cyanamide inhibited the yeast enzyme in the presence of intact rat liver mitochondria (12,13) or isolated microsomes (13), whereas, in the absence of these subcellular fractions, cyanamide was not inhibitory.

In this communication, we present evidence for the role of catalase in the conversion of cyanamide to a yet unidentified metabolite, the

Abbreviations: ED_{50}, dose giving one-half of the effective maximal response; 3-AT, 3-amino-1,2,4-triazole; N.S., nonsignificant.

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latter being the actual inhibitor of aldehyde dehydrogenase. The elevation of blood acetaldehyde was monitored to assess the relative conversion of cyanamide to its active metabolite *in vivo*. Cyanamide activation *in vitro* was estimated by following the inhibition of yeast aldehyde dehydrogenase with time.

MATERIALS AND METHODS

Yeast aldehyde dehydrogenase, bovine liver catalase, NAD^+, cyanamide and 3-AT were purchased from Sigma Chemical Company (St. Louis, MO). Glycerol was treated with sodium borohydride and then redistilled to remove aldehyde impurities. All other chemicals used were of reagent grade.

The *in vivo* experiments were conducted using male, overnight fasted rats (170-220 g) of Sprague-Dawley descent (BioLab Corp., St. Paul, MN). Each experiment was carried out on two separate days using two animals per set each day for a total of four animals per group. The basic protocol is described in the legend to Table I. The 0.22 mmol/kg cyanamide dose used is equal to twice the ED_{50} for the cyanamide induced elevation of blood acetaldehyde in the rat (13). The 1.0 g/kg dose of 3-AT inhibits rat liver catalase activity 90 percent when measured three hours after injection (14).

The rats were sacrificed by stunning and blood for acetaldehyde and ethanol measurements was collected by open chest cardiac puncture. The livers were perfused *in situ* with 20 ml of isotonic saline, then excised and a 25 percent homogenate was prepared with a 0.25 M sucrose-0.1 mM EDTA solution, pH 7.5, and stored at 0°C until analyzed. All samples were assayed on the day of the experiment.

Hepatic catalase activity was measured using a Yellow Springs Oxygen Monitor equipped with a Clark oxygen electrode essentially as previously described (15). The reaction cell was temperature controlled and maintained at 25°C. A 0.01 M potassium phosphate buffer, pH 7.15, (1.7 ml) was deoxygenated in the reaction cell with N_2. Hydrogen peroxide (7.6 µmol in 10 µl) was added to the deoxygenated buffer at zero time and baseline O_2 formation was recorded. The liver homogenate was diluted either 100- or 500-fold with 0.1 percent Triton X-100 in 0.01 M potassium phosphate buffer, pH 7.15. Then at 1 min, a 25 µl aliquot of the diluted liver homogenate was added. The difference between the rate of O_2 formation before and after the addition of liver homogenate was taken as the actual reaction rate. Catalase activity is expressed in units of natoms O_2 formed per min per µg protein. Protein was determined by the method of Lowry *et al.* (16) with bovine serum albumin as the standard.

Blood acetaldehyde and ethanol levels were measured by head space gas chromatography (17). Immediately after blood was collected, duplicate 0.2 ml aliquots were added to 20 ml serum vials containing 1.0 ml of 5.0 mM sodium azide and 0.8 µmol of n-propyl alcohol (internal standard). The vials were capped, frozen on Dry Ice and kept frozen at -78°C until assayed. In this procedure, artifactual formation of acetaldehyde from ethanol in blood is inhibited by sodium azide (18).

Partial purification of the cyanamide activating enzyme was carried out using fresh, saline perfused livers from six overnight fasted, male rats (200-250 g). The livers were minced and homogenized in 0.25 M sucrose-0.1 mM EDTA, pH 7.5. Following the standard differential centrifugation procedure, the mitochondria were washed once with 0.25 M sucrose-0.1 mM EDTA and twice with 0.25 M sucrose. After the final wash, the mitochondrial pellet was resuspended with 20 mM potassium phosphate buffer, pH 7.5 and the suspension was kept at 0°C for 20 min. The lysed mitochondria were then sonicated in an ice bath using six 10

sec bursts with a Branson Sonifier (Stamford, CT) at a #3 setting. The mitochondrial membranes were removed by centrifugation (100,000 x g for 1 hour). The soluble protein preparation was fractionated using solid ammonium sulfate. The ammonium sulfate fractions were dialyzed overnight against two three-liter volumes of 20 mM potassium phosphate buffer (pH 7.5) and stored at -20°C until assayed.

The activity of the cyanamide activating enzyme was estimated using a two-step assay system. The primary reaction contained one of the mitochondrial fractions or purified catalase, 1.0 mM NAD^+, 100 mM potassium phosphate buffer (pH 7.5) and 1.0 mM cyanamide in a final volume of 0.1 ml. The reaction was initiated by the addition of the cyanamide activating enzyme followed by aldehyde dehydrogenase 30 sec later and the mixture was incubated for 10 min at 38°C. At 10 min, a 20 µl aliquot of the primary incubation mixture was removed and added directly to a secondary reaction mixture containing 0.5 mM NAD^+, 1.0 mM EDTA, 30% glycerol and 90 mM potassium phosphate buffer (pH 8.0) in a final volume of 1.0 ml. This secondary reaction was initiated by the addition of benzaldehyde (0.6 µmol) and the remaining yeast aldehyde dehydrogenase activity was determined spectrophotometrically by following the increase in absorbance at 340 nm. When yeast aldehyde dehydrogenase and cyanamide were omitted from the primary incubation, no detectable mitochondrial aldehyde dehydrogenase activity was observed in the secondary reaction mixture.

The results are expressed as mean ± S.E.M. of triplicate samples unless indicated otherwise. The analyses of variance were determined using the Student's t-test. P values of < 0.05 were accepted as significant.

RESULTS

Preliminary characterization of the cyanamide activating enzyme, including its subcellular localization (13), enzyme stability, and apparent lack of cofactor requirement, suggested that the cyanamide activating enzyme was catalase.

This hypothesis was tested by pretreating a group of rats with the catalase inhibitor, 3-AT, followed by cyanamide and ethanol. The blood acetaldehyde and ethanol levels and total hepatic catalase activity of these animals were compared with other animal groups given cyanamide, 3-AT or isotonic saline before ethanol. Administration of cyanamide increased blood acetaldehyde 90-fold compared to the saline control group (Table I). 3-AT pretreatment attenuated this cyanamide-induced elevation of blood acetaldehyde more than 90 percent. 3-AT alone had no significant effect on blood acetaldehyde levels. In these same animals, total hepatic catalase was inhibited 90 percent with a 3-AT dose of 1.0 g/kg (12 mmol/kg). However, cyanamide also inhibited hepatic catalase *in vivo* (Table II). At the dose used (0.22 mmol/kg), cyanamide decreased hepatic catalase activity 75 percent.

The attenuation of cyanamide-induced acetaldehydemia by 3-AT was dose dependent (Fig. 1). Pretreatment of the animals with a 1.0 g/kg dose of 3-AT almost completely blocked the cyanamide effect on blood acetaldehyde levels. As the 3-AT dose was reduced, its attenuating

TABLE I

Effect of 3-AT on the Elevation of Ethanol-Derived Blood Acetaldehyde by Cyanamide in the Rat

Animal Group*	Blood Acetaldehyde		Blood Ethanol** (mM)
	μM	Elevation relative to control	
Saline Control	14.2 ± 3.2	1.0	45.7 ± 1.0
Cyanamide (0.22 mmol/kg)	1284 ± 76	90.4	46.0 ± 2.1
3-AT (1 g/kg), Cyanamide	78.6 ± 22.4	5.5	43.1 ± 2.6
3-AT	12.4 ± 2.5	0.9	45.7 ± 1.7

*Each group of four rats was administered saline or 3-AT at zero time, saline or cyanamide at 3 hours, and ethanol (2 g/kg) at 4 hours. All drugs were administered i.p. The animals were sacrificed 1 hour after ethanol.

**Blood ethanol values of experimental groups were not significantly different from the saline control.

effect on cyanamide-induced blood acetaldehyde decreased. The hepatic catalase activity measured concommitantly paralleled the blood acetaldehyde values (Fig. 1). Although a comparison of the blood acetaldehyde levels with hepatic catalase activity was complicated by the significant inhibition of this enzyme by cyanamide itself (in addition to its inhibition by 3-AT), a positive correlation between blood acetaldehyde levels and hepatic catalase activity was observed (Fig. 2).

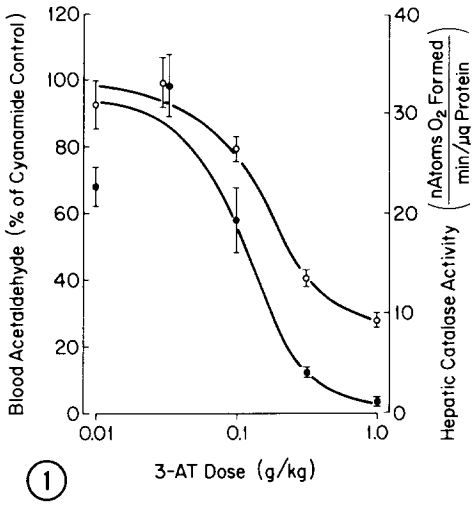
In previous *in vitro* studies (12,13), we showed that the rat liver mitochondrial fraction contained significant cyanamide activation activity. Partial purification of this cyanamide activating enzyme is shown in Table III. Intact mitochondria were osmotically disrupted and then sonicated and carried through ammonium sulfate fractionation. The bulk of the recovered activity was found in the 50-65% ammonium sulfate fraction.

TABLE II

Inhibition of Total Hepatic Catalase Activity by Cyanamide and 3-AT*

Animal Group	Hepatic Catalase Activity		P Value
	nAtoms O_2 formed min/μg protein	Percent of control	
Saline Control	170.8 ± 10.2	100	-
Cyanamide (0.22 mmol/kg)	40.9 ± 5.3	23.9	< 0.001
3-AT (1.0 g/kg), Cyanamide	14.6 ± 0.3	8.5	< 0.001
3-AT	15.6 ± 2.1	9.1	< 0.001

*Data was obtained from the same set of animals shown in Table I.



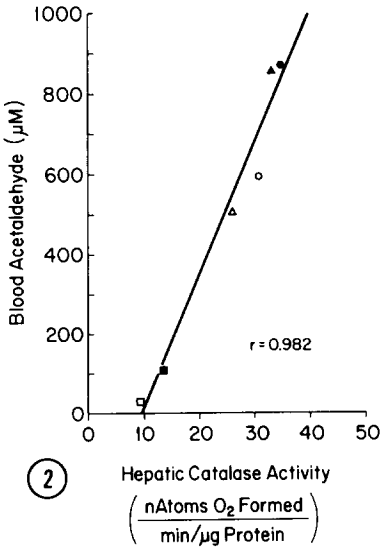


FIGURE 1. Dose response curves for the attenuation of the cyanamide induced elevation of blood acetaldehyde (●) and for the inhibition of total hepatic catalase (○) activity by 3-AT in the rat. The experimental details were as described in Table I, except for the dose of 3-AT which is indicated above. The blood acetaldehyde values for the saline control (no added 3-AT or cyanamide) and cyanamide control (no added 3-AT) were 9.7 ± 1.9 and 872 ± 109 µM, respectively; whereas, their corresponding hepatic catalase activities were 189.7 ± 15.5 and 34.7 ± 2.7 natoms O_2 formed per min per µg protein. The blood ethanol levels for the experimental groups did not differ significantly from the saline control group which was 46.3 ± 0.7 mM.

FIGURE 2. Correlation of mean blood acetaldehyde levels with mean hepatic catalase activity for rats given various single doses of 3-AT and a single 0.22 mmol/kg dose of cyanamide (replot of data from Figure 1). The doses of 3-AT were (●) 0.00, (○) 0.01, (▲) 0.032, (▽) 0.1, (■) 0.32 and (□) 1.0 g/kg.

Purified bovine liver catalase also catalyzed the conversion of cyanamide to its active form (Table IV). The formation of an active cyanamide metabolite was assessed by determining the degree of inhibition of yeast aldehyde dehydrogenase. Inhibition of this enzyme required both catalase and cyanamide and increased with catalase concentration. NAD^+ was also required for inhibition of aldehyde dehydrogenase (data not shown). Sodium azide effectively blocked the activation of cyanamide catalyzed by bovine catalase (Table IV) and by the 50-65% ammonium sulfate mitochondrial fraction (data not shown). Similar results were obtained with ascorbate (4 mM) as the hydrogen peroxide source or with hydrogen peroxide (4 mM) added directly (data not shown). The presence of hydrogen peroxide--added exogenously or produced enzymatically--inhibited aldehyde dehydrogenase and this inhibition was not blocked by sodium azide. The apparent increase in aldehyde dehydrogenase activity with catalase in the absence of added cyanamide (Table IV) was likely due to the removal of hydrogen peroxide by catalase.

TABLE III

Partial Purification of the Cyanamide Activating System from Rat Liver Mitochondria

Fraction	Total Protein (mg)	Relative* Specific Activity	Total Activity (percent)
Osmotically Disrupted Mitochondria	128.1	1	100
Soluble Fraction after Sonication	111.4	0.74	64
35-50% Ammonium Sulfate	44.5	0.16	5.6
50-65% Ammonium Sulfate	3.4	12.9	34.5
> 65% Ammonium Sulfate	17.9	< 0.1	< 1

*The specific activity equals the amount of mitochondrial protein (μg) required to catalyze the activation of cyanamide (1.0 mM) and cause a 50 percent inhibition of yeast aldehyde dehydrogenase under the experimental conditions described under Methods. Fifty percent inhibition was estimated from plots of log of the percent inhibition versus mitochondrial protein for each fraction. For the first fraction, i.e., the osmotically disrupted mitochondrial fraction, 1.62 μg protein was required for 50 percent inhibition. The relative specific activity of the first fraction was set at one and the specific activities of the remaining fractions were normalized against this value.

DISCUSSION

The major metabolic pathway for cyanamide in the rat, rabbit, dog and man (19) involves acetylation catalyzed by an acetyl-S-CoA dependent hepatic N-acetyltransferase yielding N-acetylcyanamide which is excreted in the urine. The lack of a direct inhibition by cyanamide of purified aldehyde dehydrogenase enzymes (1,5,11,12 and Table IV) and the failure of N-acetylcyanamide to inhibit aldehyde dehydrogenase even in the presence of the cyanamide activating enzyme (13), suggested that a second--albeit minor--pathway for cyanamide metabolism must exist. Our search for such a pathway led us to catalase.

TABLE IV

The Conversion of Cyanamide to an Inhibitor of Aldehyde Dehydrogenase by Bovine Liver Catalase*

Cyanamide (1.0 mM)	Catalase (μg)	NaN_3 (0.5 mM)	Yeast Aldehyde Dehydrogenase Activity (percent of control)	P Value
-	0.00	-	100.0 ± 4.1	-
+	0.14	-	30.2 ± 2.4	< 0.001
+	0.50	-	2.2 ± 0.7	< 0.001
+	0.00	-	100.6 ± 2.8	N.S.
-	0.14	-	129.8 ± 3.1	< 0.01
+	0.14	+	84.6 ± 0.7	< 0.05
+	0.50	+	91.3 ± 1.3	N.S.

*Experimental details were as described under Methods except that 0.6 units of glucose oxidase and 10 mM glucose were included in the primary incubation as the hydrogen peroxide source.

Pretreatment of rats with the catalase inhibitor, 3-AT, blocked the cyanamide induced elevation of blood acetaldehyde (Table I) implicating catalase in the cyanamide activation mechanism. The degree of attenuation of the blood acetaldehyde levels was dependent on the dose of 3-AT administered (Fig. 1). The attenuation of blood acetaldehyde by 3-AT (1 g/kg) was accompanied by a 90 percent inhibition of hepatic catalase activity. However, hepatic catalase was also strongly inhibited by cyanamide itself (Table II) and, therefore, the decreased hepatic catalase activity was the result of combined inhibitory effects of 3-AT and cyanamide. Nevertheless, a strong positive correlation between hepatic catalase activity and the level of blood acetaldehyde was evident (Fig. 2).

Partial purification of the cyanamide activating enzyme demonstrated that the bulk of the activity resided in the 50-65% ammonium sulfate fraction (Table III). Recently, Marchner and Tottmar (20) showed that the aldehyde dehydrogenase in the 40-60% ammonium sulfate fraction of rat liver mitochondria was inhibited by cyanamide. The co-purification of the cyanamide activating enzyme with aldehyde dehydrogenase likely accounts for this observation, rather than, as suggested, a direct inhibition of aldehyde dehydrogenase by cyanamide itself. Indeed, the aldehyde dehydrogenase in the 40-60% ammonium sulfate fraction of rat liver mitochondria has been separated from the cyanamide activating enzyme by affinity chromatography (H. Weiner, personal communication).

The role of catalase in the bioactivation of cyanamide was confirmed using the purified bovine liver enzyme. Bovine catalase (0.5-2.0 pmol) in the presence of hydrogen peroxide converted cyanamide to its active form (Table IV). The cyanamide activation reaction catalyzed by bovine catalase and the 50-65% ammonium sulfate fraction of rat liver mitochondria was inhibited by sodium azide. Whether catalase is the only enzyme able to catalyze this biotransformation of cyanamide *in vivo* is not known. However, the high correlation between the cyanamide induced elevation of blood acetaldehyde and hepatic catalase activity (Fig. 2) and the effective blockade of the former by 3-AT *in vivo* (Table I) suggest that catalase may well be the only enzyme involved.

The preparation of subcellular liver fractions by conventional differential centrifugation techniques yields a mitochondrial fraction containing mitochondria, lysosomes and peroxisomes (21). The latter is known to contain significant catalase (21,22). Both mitochondrial and peroxisomal catalase are likely involved in cyanamide activation *in vivo* and *in vitro*.

Additional studies are in progress a) to further characterize the role of catalase in the bioactivation of cyanamide, b) to describe the

inhibition of catalase by cyanamide and c) to identify the active cyanamide metabolite.

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