Biochemical Pharmacology, Vol. 25, pp. 1101–1105. Pergamon Press, 1976. Printed in Great Britain.

EVIDENCE FOR ALTERED HEPATIC CATALASE MOLECULES IN ALLYLISOPROPYLACETAMIDE-TREATED MICE*

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(*Received* 8 *March* 1975; *accepted* 22 *August* 1975)

Abstract—Treatment of C57BL/6J male mice with allylisopropylacetamide (AIA) produced a decline in hepatic catalase activity. However, a large fraction (50 per cent of the enzyme) is unaffected by treatment with the drug. The enzyme purified from AIA-treated mice is spectrally, electrophoretically and immunologically indistinguishable from that prepared from saline-treated animals. Specific enzyme activity of the enzyme prepared from AIA-treated animals is approximately one-half that of enzyme prepared from saline-treated mice. These results suggest that the decline in hepatic catalase activity observed in AIA-treated mice is not solely a result of interference of the drug with hepatic heme biosynthesis, or insensitivity of a fraction of the enzyme to AIA, but that the catalase enzyme present in AIA-intoxicated mouse liver possesses some alteration in molecular structure.

Catalase (EC 1.11.1.6) is an enzyme found in a variety of tissues in aerobic organisms. The properties and characteristics of the enzyme have been recently reviewed [1]. Catalase enzyme activity is normally maintained in a steady state, such that the number of molecules destroyed per unit of time equals the number of molecules synthesized within the same time period. The enzyme is regulated in a differential manner, in that different levels of activity are present in different tissues within the same organism [2]. The level of enzyme activity is inducible to much higher levels in both rat [3, 4] and mouse liver [5] through treatment of the animals with α-*p*-chlorophenoxyisobutyrate (clofibrate) which increases the rate of enzyme synthesis, thereby increasing the amount of catalase protein [4].

One of the most interesting aspects of catalase research concerns the steady state regulation of the activity of the enzyme. Heim, Appleman and Pyfrom [6] demonstrated that 3-amino-1,2,4-triazole (3-AT) destroyed rat liver and kidney catalase, without interfering with its resynthesis, through an irreversible binding of the drug to the protein moiety of the enzyme [7]. Through an analysis of the rate of renewal of catalase activity following the destruction of enzyme activity with 3-AT, both the rates of synthesis (K_S) and degradation (K_D) of rat hepatic and renal catalase were determined [8]. These findings were subsequently expanded and thoroughly discussed [9, 10]. This method has subsequently been exploited to study the regulation of catalase activity in animals of differing genotypes [11], differing chronological ages [12], in insects [13], and in human diploid cells [14].

The effectiveness of the '3-AT method' for the determination of both K_D and K_S for catalase was originally substantiated by an alternative method. It was shown that rats treated with allylisopropylacetamide (AIA) exhibit a fall in liver catalase activity resulting from interference with heme biosynthesis in liver of treated animals [15]. The slope of this exponential decline in enzyme activity should be equal to K_D, the rate of enzyme destruction, and it was demonstrated that K_D for rat catalase activity determined directly by the AIA method was identical to that obtained indirectly from the 3-AT method [9].

In addition, however, it was observed in AIA-treated rats that about 8 per cent of the hepatic catalase was unaffected by treatment with the drug [9]. This observation apparently was not a major consideration at that time, as it did not cause difficulty as regards either the 3-AT or AIA methods of determining the K_D for catalase in rodent tissues. In the present paper we show that a much larger (50 per cent) proportion of hepatic catalase activity in C57BL/6J male mice is insensitive to treatment with AIA. Furthermore, purification of the catalase protein present in the livers of AIA-treated mice results in an enzyme preparation with a markedly lower specific enzyme activity than of that prepared from saline-treated control animals.

MATERIALS AND METHODS

C57BL/6J male mice were used throughout the course of this study. The animals were 200 days old and weighed 30–32 g at the time of sacrifice. The mice were purchased at 2 months of age and maintained in our colony, six animals per cage, at 23 ± 0.5° and approximately 50 per cent relative humidity. Lights were 'on' from 0600–1800 hr EST. Purina lab chow and tap water were available to the animals *ad lib.*

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In the initial experiments, animals were divided into four groups. Mice in Group A were injected twice daily, at 0900 and 2100 hr EST, with allylisopropylacetamide (AIA) at a dose level of 100 mg/kg body wt (20 mg AIA/ml in water). Animals in Group B were injected with 3-amino-1,2,4-triazole (3-AT) at a dose level of 1 g/kg body wt (100 mg 3-AT/ml in water). The animals immediately received another injection, which consisted of AIA as described, with succeeding injections of AIA at 12-hr intervals. Animals in Group C were injected with AIA for 2 days, twice daily. On day three and succeeding days they received treatment as described for Group B. Thus, animals in Group C were treated as were those in Group B, except that they were pretreated for 2 days with AIA. Animals in Group D received injections of water (0.3 ml) twice daily at the indicated times. All injections were given intraperitoneally.

At each of the indicated times after the initiation of pharmacological treatment, five animals per group were killed by cervical dislocation. The livers were removed, rinsed twice in ice-cold water, and blotted dry. Individual livers were weighed and homogenized in a Teflon glass homogenizer with 10 ml of water. The homogenates were filtered through two layers of fiber-glass cloth (Miracloth) into a 50-ml volumetric flask, and brought to volume with ice-cold water.

Whole homogenates were assayed for catalase activity by the spectrophotometric method previously described [12], except that the reaction temperature was 24 . Protein content was determined by the microbiuret method [16] using crystalline egg albumin standards.

Mouse liver catalase was purified from three groups of animals which had received AIA for 2 days, 3-AT (once) followed by AIA for 2 days, or saline. The catalase was purified essentially as described by Price *et al.* [9], and enzyme activity at different steps in the purification procedure was determined. Protein content was determined by a Folin method [17] using standards of crystalline egg albumin. Purified enzyme solutions were dialyzed overnight into 0.02 M sodium phosphate, pH 6.8 prior to subsequent characterization.

Absorption spectra of the purified enzymes were determined with a Cary Model 11M scanning spectrophotometer.

Purified catalase solutions were electrophoresed in 5% polyacrylamide gels with a Buchler Gel Electrophoresis Apparatus. Running pH of the system was 8.3 (boric acid Tris HCl buffer). The current applied was 2 mA per gel column, and electrophoresis was carried out for 2 hr. The resultant gels were stained in 1% Amido Schwartz B made up in 7% acetic acid and then destained in 7% acetic acid for 1 hr. Drawings of the gels were prepared immediately after destaining as some of the very faint bands tended to disappear when the gels were stored overnight in 7% acetic acid.

Sedimentation velocity constants of the purified mouse liver catalase were determined in a Beckman Model E ultracentrifuge operating at 56,000 rpm. The cells were scanned at 405 nm.

Gel diffusion plates (Ouchterlony) were prepared with 1% Difco Noble Agar. The purified mouse catalases were placed in the outer wells of the gel. Antiserum to purified rat hepatic catalase was placed in the inner well. The gels were photographed after 24 and 48 hr of incubation at room temperature. Antiserum against rat hepatic catalase was prepared in the following manner. Catalase was purified as described by Price *et al.* [9]. The O.D. 407:276 ratio of the purified preparation was 1.06. Young adult male New Zealand White rabbits were injected subcutaneously along the flanks with 1 mg of purified catalase in complete Freund's adjuvant. The animals received an intramuscular booster of 1 mg of catalase after 3 weeks. Blood was collected 2 weeks after the booster injection by cardiac puncture. Whole serum was collected by centrifugation, heat-treated at 56 for 30 min, and stored frozen in small aliquots.

RESULTS

Treatment of male C57BL/6J mice with AIA produces a decrease in hepatic catalase activity to one-half the initial activity 48 hr after initiation of treatment (Fig. 1, line A). Repeated injections of AIA for longer periods of time produce no additional effect. Injection of mice with 3-AT and AIA produces a fall in catalase activity, followed by a return to one-half the initial level of enzyme activity (Fig. 1, line B). Identical results were obtained when the mice were pretreated with AIA for 48 hr prior to treatment with 3-AT and AIA (Fig. 1, line C).

It seemed reasonable to assume that these results represented a species difference between the mouse and the rat as regards the dose-response of hepatic catalase activity to AIA treatment. Doubling the dose (200 mg/kg body wt, twice daily), however, resulted in identical results, while 300 mg/kg body wt of AIA twice daily was lethal to the mice.

Thus, while a small fraction (8 per cent) of hepatic catalase activity in the male Buffalo rats is insensitive

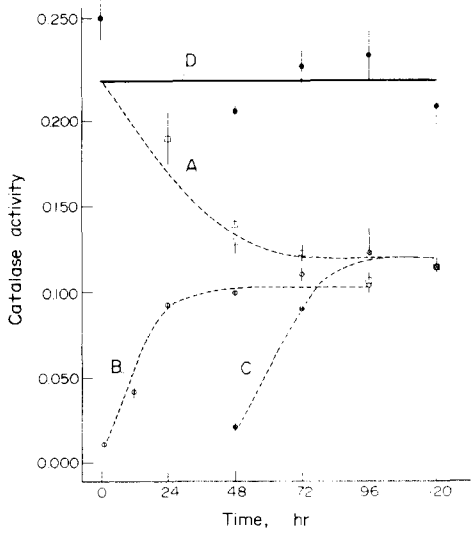

Fig. 1. Time course of changes in C57BL/6J male mouse hepatic catalase activity following treatment with water (●), allylisopropylacetamide (□) or allyisopropylacetamide and 3-amino-1,2,4-triazole (○,).

to AIA treatment [9], approximately 50 per cent of the enzyme activity in C57BL/6J mouse livers appears unaffected by the drug. These results indicate that AIA only partially interferes with hepatic heme biosynthesis in the mouse, assuming that AIA produces effects in the mouse identical to those which are produced by the drug in rats. An alternative explanation for these findings is that mouse liver produces altered molecules (e.g., only partially active or totally inactive enzyme) in the presence of the drug.

Therefore, hepatic catalase was purified from saline-treated and AIA-treated mice, and partially characterized by several physicochemical methods. There was no obvious difference in the u.v.–visible spectra of catalases prepared from saline-treated and AIA-treated mice (Fig. 2). Assuming, for the moment, that the criteria for purity set down for purified rat liver catalase [9] are applicable to purified mouse liver catalase, both preparations were at least 99 per cent pure (407:276 ratio for saline-treated was 1.09, while the 407:276 ratio for AIA-treated was 1.05). The strong Cotton effect in the Soret band in both preparations was destroyed by treatment with 8 M urea, with a subsequent shiftt from 407 to 368 nm. This shift was accompanied by a 60–65 per cent loss in magnitude of absorption. There was little effect of treatment with 8 M urea upon either the position or magnitude of the peak corresponding to protein (276 nm).

The results obtained upon electrophoretic separation of the two purified catalase preparations are shown in Fig. 3. There were no great differences in electrophoretic mobility of the purified catalases, suggesting that the loss in catalase activity in AIA-treated mice is not a result of the production of subunits, with concomitant loss of activity. Both preparations are characterized by a slow-moving major band and several more rapidly migrating minor components. Dilution of the preparations followed by subsequent electrophoresis resolves the major band into four components, which quite possibly represent different oxidation states of the purified enzyme as appears to be the case with 'isozymes' of beef liver catalase [18]. The minor bands are extremely faint to the unaided eye, and probably represent only a very small percentage of the total material.

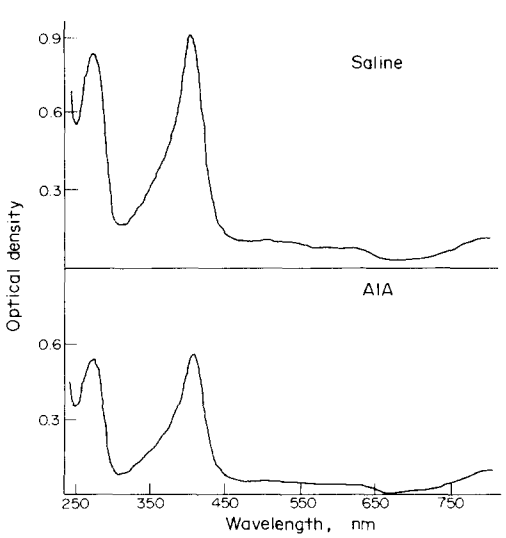

Fig. 2. u.v.–visible spectra of hepatic catalase purified from C57BL/6J male mice treated twice daily for two successive days with allylisopropylacetamide or saline.

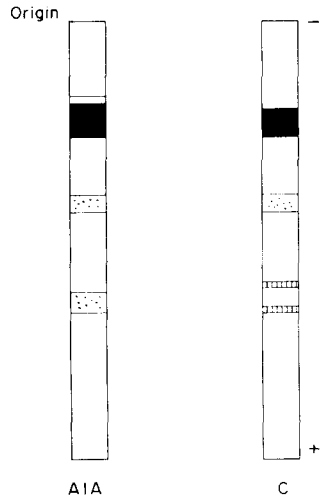

Fig. 3. Electrophoretograms of hepatic catalase purified from C57BL/6J male mice following treatment for two successive days with allylisopropylacetamide or saline.

Gel diffusion plates reveal that purified mouse liver catalase is immunologically similar to purified rat liver catalase, as shown by the heavy precipitin band produced by the interaction between mouse liver catalase and rat liver catalase antiserum (Fig. 4). There is no indication from Fig. 4 and from other plates not shown that there are any immunological differences between the hepatic catalase purified from AIA-treated mice and enzyme prepared from control animals.

These results obtained with purified enzyme preparations are consistent with the motion that AIA interferes with liver heme biosynthesis to such an extent that the intoxicated livers are able only to produce one-half of the catalase protein which is normally produced. Table 1 shows, however, that the specific enzyme activity of catalase purified from AIA-treated mice is approximately one-half that of enzyme purified from control animals. This difference is manifest

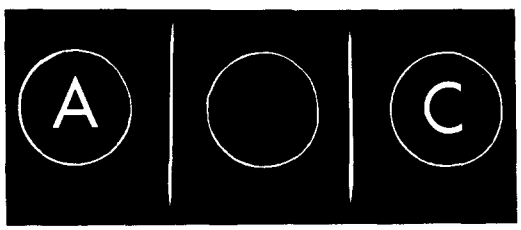

Fig. 4. Gel diffusion of hepatic catalase purified from C57BL/6J male mice following treatment for two successive days with allylisopropylacetamide or saline. A—catalase prepared from allylisopropylacetamide-treated animals; C—catalase prepared from saline-treated animals; Center well—rat hepatic catalase antiserum.

Table 1. Recovery of hepatic catalase activity from C57BL/6J male mice at different stages of purification following treatment with saline, allylisopropylacetamide or 3-amino-1,2,4,triazole and allylisopropylacetamide.

Treatment	Liver wt. g	Sample	Total vol. ml	Units/ml	Total units	Units/mg protein
		Homogenate	460.0	8.44	3882	0.50
		S_1	391.0	8.00	3128	6.26
Saline	47.2	S_3	18.1	127.92	2315	23.35
		S_0	1.2	1101.51	1322	228.34
		Homogenate	692.0	5.22	3612	0.33
		S_1	593.0	4.00	2372	3.46
AIA and 3-AT	73.3	S_3	26.0	76.59	1991	14.10
		S_0	1.4	476.36	667	146.15
		Homogenate	650.0	6.20	4030	0.33
		S_1	550.0	3.48	1914	2.40
AIA	65.5	S_3	25.5	62.16	1585	13.78
		S_0	1.3	438.85	570	127.83

S_1, S_3 and S_0 refer to steps in the purification procedure described by Price *et al.* (1962).

throughout the course of the purification of the enzyme from AIA-treated animals (Table 1). It appears, therefore, that the catalase molecules found in AIA-treated mouse liver are catalytically distinct from those found in control animals, even though these differences are not demonstrated by spectral, electrophoretic or immunological methods.

We have noted a slight difference in sedimentation velocity constants between the two purified catalases. The velocity constants have been calculated as follows: AIA, $S_{w,\,20} = 11.2$, saline, $S_{w,\,20} = 11.6$. We do not know at the present time if this slight difference is significant, inasmuch as it represents single determination of sedimentation velocity constants. It does suggest, however, that the catalase molecules purified from AIA-treated mice may differ in size and/or shape which are not detectable by a number of other methods.

DISCUSSION

The role of catalase in any organism is unknown. It has been suggested that the enzyme is superfluous, a fossil enzyme [19]. This notion is indeed a distinct possibility as studies from our laboratory have shown that experimental destruction of catalase activity in *Drosophila melanogaster* with 3-AT has no effect upon adult survival [13]. It is possible, however, that the presence of the enzyme is obligatory during preadult development, or that there are other enzyme systems which are able to perform the functions of catalase when catalase activity is absent.

In any case, studies of the regulation of catalase activity in a variety of tissues, and under various physiological states, are potentially of signal importance in that they may yield information about other less readily studied steady-state enzymes, as well as provide a method to determine the effects of different physiological states (e.g., age, species or pharmacological treatment) upon the regulation of activity of at least one enzyme.

The results of this study clearly show that the response of mouse liver catalase activity to treatment with AIA differs from that observed in the rat. It appears from the results of this study that treatment of mice with AIA results in the formation of a molecule with altered enzyme activity possibly coincident with an altered sedimentation velocity. Inasmuch as we have not, as yet, studied the effects of AIA upon heme biosynthesis in mouse liver, a fraction of the decline in catalase activity in treated animals quite possibly reflects interference of heme biosynthesis by the drug.

Price *et al.* [9] discussed the AIA-resistant catalase observed in male Buffalo rats. They suggested that this activity might be a property of catalase which is distributed in cells which are resistant to AIA. This is a distinct possibility since a number of rodent tissues (e.g. kidney) as well as certain hepatomas are sensitive to 3-AT, but not to AIA [20]. In this connection we have found that concentrations of AIA in the food medium of *Drosophila* up to the lethal limit (10 mg/ml) have no effect upon *Drosophila* catalase activity (unpublished data).

Certain hepatic cell types may be insensitive or less sensitive to AIA than are others. Van Berkel [21] has shown that there is a much higher catalase activity in rat hepatic parenchymal cells than is found in reticulo-endothelial (Kupffer) cells. If Kupffer cells are insensitive to treatment with AIA, their contribution to total hepatic catalase activity would be unaffected by the drug. Although these cells make up approx 30 per cent of the rat liver mass [22], it is not known what proportion of the liver in the mouse is represented by reticulo-endothelial cells. Furthermore, this tentative suggestion must be considered in light of the fact that C57BL/6J mice exhibit a regulatory mutation which affects hepatic catalase activity [11]. It is not known whether the mutation affects parenchymal and non-parenchymal cells to the same extent. This suggestion does not explain the apparent presence of altered hepatic catalase molecules in AIA-treated mice. It is possible, however, that there are normally two types of catalase with differing enzyme activities elicited in mouse liver, and that AIA preferentially interferes with the synthesis of one form.

We suggest that AIA produces manifold effects in experimental animals. Treatment with the drug results in a decline in all heme-containing molecules in rat liver, through its effect on heme biosynthesis [15].

However, the drug also specifically degrades microsomal cytochrome P_{450}, a hemeprotein, through degradation of the molecule within the microsomal membranes [23]. Finally, the results of this study suggest that the drug specifically alters the structure of catalase, thereby altering its activity, or selects from several forms of hepatic catalase one representative which possesses a distinct specific enzyme activity. This general scheme allows for the interference of heme biosynthesis which produces a decline in catalase activity in AIA-treated mice, as well as for production of altered molecules with a decreased enzyme activity in the treated animals. In addition, a manifold action of AIA also permits an explanation for the specific effect of the drug upon microsomal cytochrome P_{450} [23]. The observed decline in hepatic enzyme activity observed in the AIA-treated mice would then be a result of two distinct, yet simultaneous, processes, both of which effectively produce a decreased enzyme activity.

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