PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 158, 398–401 (1978)

Cinnabarinate Synthase Activity in Normal and Acatalasemic Mice[1] (40212)

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3-Hydroxyanthranilic acid (HAA) is a carcinogen which is oxidatively dimerized by an enzyme known as cinnabarinate synthase (1) to another carcinogen, cinnabarinic acid (2-amino-3-oxo-3H-phenoxazine-1,9-dicarboxylic acid) (2, 3). Savage and Prinz (4) recently demonstrated that the reaction can be carried out by catalase in the presence of manganous ion, and they suggested that, in fact, the enzyme known as cinnabarinate synthase may actually be catalase.

We have produced in this laboratory strains of mice mutant with respect to blood and tissue catalase (5). The acatalasemia gene Cs^b (6) has been introduced into highly inbred strains of C3H and C57BL mice, and the availability of these mutant substrains offered an excellent opportunity to confirm, in a very different manner, the conclusions of Savage and Prinz (4). I report here that cinnabarinate synthase activity does indeed parallel catalase activity in the several respects tested. The role of certain metals in the reaction is also examined.

Materials and methods. Mice used were C3H/Cs^a, C3H/Cs^b, C57BL/Cs^a, and C57BL/Cs^b. Cs^a indicates the normal catalase, "wild type" substrain; Cs^b indicates that the acatalasemic gene has been introduced. In mice carrying Cs^b, blood catalase activity is reduced to 1–5% of normal (5, 7); the catalase activity of liver and other solid tissues is unstable and, if assayed at 37°, shows from 10 to 40% of normal activity (7, 8).

3-Hydroxyanthranilic acid was purchased from Sigma Chemical Company. 3-Amino-1,2,4-triazole (AT) was purchased from ICN—K and K Laboratories. Cinnabarinate synthase assays were performed by the method of Savage and Prinz (4), with the reaction being halted by H_3PO_4. Mixtures were incubated for 30 min at 37°. As suggested by Savage and Prinz, mixtures containing active extracts were simultaneously incubated with mixtures containing boiled extracts, and the difference in A_{540} is considered a measure of activity. The incubation time of 30 min was taken directly from Savage and Prinz (4). It is, however, an arbitrary time; as in the case of the catalase assay (9), the reaction rate decreases with time.

Catalase was assayed at 37° by the perborate method (9). The assays of liver enzyme activity were done on whole liver homogenates, prepared at 2% with cold water, then further diluted with an equal volume of 1% Triton X-100 (10). Assays of enzyme activity in blood were done on 2% lysates of whole blood. Aliquots of the same extracts were used for assay of the two enzymes, although it was sometimes necessary to dilute further with water for catalase assays. Unless otherwise specified, 10^{-3} *M* $MnCl_2$ (final concentration) was included in all cinnabarinate synthase assays (4). Other metal salts, when used, were also present at a final concentration of 10^{-3} *M* unless otherwise noted.

Results. Table I shows that, in both C3H and C57BL mouse strains, the presence of the acatalasemia gene, Cs^b, decreases both cinnabarinate synthase and catalase activities to approximately the same extent in liver. In blood, cinnabarinate synthase activity is also decreased, but to a lesser extent than is catalase activity.

The effect of temperature of incubation was tested. The livers of normal and acatalasemic mice were assayed for cinnabarinate synthase activity, using both a 30-min incubation at 37°, and a 30-min incubation in an ice bath. Table II indicates that while the liver cinnabarinate synthase activity of the two strains differs if assayed at 37°, the activity of the same extracts is not significantly different if assayed at ice bath temperature.

The effect of the catalase inhibitor, 3-amino-1,2,4-triazole, was tested simultaneously on the catalase and cinnabarinate synthase activities of liver and blood (Table III), and again the activities of the two enzymes

[1] Work supported by the U.S. Department of Energy.

0037-9727/78/1583-0398$01.00/0

TABLE I. CINNABARINATE SYNTHASE AND CATALASE ACTIVITIES OF NORMAL AND ACATALASEMIC MICE.[a]

		Cinnabarinate synthase			Catalase		
Tissue	Strain	Cs^a	Cs^b	Ratio	Cs^a	Cs^b	Ratio
Liver	C3H	1.15 ± 0.02	0.25 ± 0.06	4.6	1225 ± 35	283 ± 9	4.3
	C57BL	0.91 ± 0.03	0.25 ± 0.01	3.6	1145 ± 15	294 ± 5	3.9
Blood	C3H	1.24 ± 0.03	0.88 ± 0.03	1.4	109 ± 3	1.9 ± 0.2	57
	C57BL	0.96 ± 0.07	0.44 ± 0.07	2.2	149 ± 3	7.0 ± 0.2	21

[a] Data are mean and standard error of the mean for groups of three individual mice. Cs^a indicates normal catalase, "wild type" mouse; Cs^b is the acatalasemic mutant. Catalase activities are expressed as perborate units (9) per gram liver or per ml whole blood. Cinnabarinate synthase activities are expressed in arbitrary units, the difference in A_{450} between mixtures containing active extracts and those containing boiled extracts (4).

TABLE II. EFFECT OF ASSAY TEMPERATURE ON LIVER CINNABARINATE SYNTHASE ACTIVITY.[a]

Activity	Cs^a	Cs^b	Ratio	t	p
Assayed at 37°	1.40 ± 0.03	0.24 ± 0.02	5.8	30.33	0.001
Assayed in ice	0.10 ± 0.01	0.06 ± 0.03	1.7	1.33	N.S.

[a] Data are mean and SEM for groups of three normal or acatalasemic C3H female mice. Units are described in Table I.

responded comparably.

The specificity of manganese for the cinnabarinate synthase reaction was tested (Table IV); at 10^{-3} *M* metal ion, only manganese produced more color with active enzyme source than with the same preparation after boiling. Copper produced the same intense color whether the enzyme source was boiled or not. It was found that 10^{-3} *M* copper alone, in the absence of any tissue or enzyme preparation, catalyzed the oxidative dimerization so effectively as to mask any copper-enzyme interaction. If the concentration of the copper is sufficiently reduced, however, it could be demonstrated that this interaction does occur (Table V). Table V also indicates that Cu is actually effective at an order of magnitude lower concentration than is Mn.

Discussion. The introduction of the acatalasemia locus, Cs^b, is generally considered to include no modifications other than in catalase biosynthesis. The conclusion of Savage and Prinz (4), that cinnabarinate synthase is identical with catalase is therefore bolstered by the fact that the introduction of Cs^b also inhibits the cinnabarinate synthase activity of blood and liver.

In liver, the two enzyme activities are approximately equally affected by Cs^b. In blood, cinnabarinate synthase activity is very much less affected than is catalase activity. This is presumably due to the fact that hemoglobin has been shown to have a cinnabarinate synthase activity (11). The presence of large and equal amounts of hemoglobin in Cs^a and Cs^b blood masks the magnitude of the effect on the catalase molecule.

It has been shown (7, 8) that the liver catalase of acatalasemic mice is unstable and, if assayed at 37°, shows considerably less activity than that of normal mice; on the other hand, the liver catalase activity of the two strains is approximately equal if measured at ice bath temperature. Table II shows that cinnabarinate synthase activity in acatalasemic liver is comparably heat-labile, again arguing for the identity of the two enzymes.

Injected aminotriazole is known to inhibit the catalase of the liver and other solid tissues, but not that of the blood (12–14). This differential effect was shown by Margoliash and Schejter (15) to be due to the existence in erythrocytes of a dialyzable sulfhydryl catalase donor. The fact that AT has the same differential effect on the cinnabarinate synthase activity of blood and liver that it does on catalase activity is further suggestive evidence for the identity of the enzymes carrying out the two reactions.

The question of metal-heme interaction is of particular interest. The cinnabarinic acid generated by Mn or Cu in the presence of boiled liver homogenate (Table IV) or boiled preparation of pure beef liver catalase (Table V) is due solely to the metal present; the boiled enzyme sources contribute no additional color, while unheated enzyme does. It therefore appears that the oxidative dimerization of 3-hydroxyanthranilic acid can be carried out by Mn^{2+} or Cu^{2+} alone, but the

TABLE III. EFFECT OF 3-AMINO-1,2,4-TRIAZOLE (AT) ON BLOOD AND LIVER CATALASE AND CINNABARINATE SYNTHASE ACTIVITIES.[a]

		Cinnabarinate synthase		Catalase	
Tissue	Treatment	Activity	Effect of AT	Activity	Effect of AT
Liver	Saline	1.42 ± 0.03		1313 ± 199	
	AT	0.51 ± 0.11	−64%	138 ± 31	−89%
Blood	Saline	1.33 ± 0.06		110 ± 2	
	AT	1.34 ± 0.04	+1%	110 ± 3	0

[a] Mice were injected intraperitoneally either with 1000 mg AT/kg, or with 10 ml/kg of 0.9% NaCl and were sacrificed one hour later. Data are mean and SEM for groups of three C3H mice. Units are described in Table I.

TABLE IV. EFFECT OF METAL IONS ON CINNABARINATE SYNTHASE ACTIVITY.[a]

	A_{450}		
Metal salt	Active extract	Boiled extract	Difference
None	0.059	0.078	−0.019
$CaCl_2$	0.066	0.087	−0.021
$CdCl_2$	0.087	0.116	−0.029
$Cu(OAc)_2$	1.862	1.893	−0.031
$Fe(NH_4)_2(SO_4)_2$	0.027	0.085	−0.058
$FeCl_3$	0.501	0.539	−0.038
$MgCl_2$	0.076	0.099	−0.023
$NiCl_2$	0.066	0.111	−0.045
$ZnCl_2$	0.074	0.101	−0.027
$MnCl_2$	1.620	0.626	+0.994

[a] All metal salts were present in a final concentration of 10^{-3} *M*. Enzyme source was homogenate of C3H/Cs[a] mouse liver. Before obtaining the readings of Table IV, all final solutions were passed through Millipore filters (0.45 micra), because of a faint haze in the solutions, particularly those containing boiled extract.

TABLE V.[a]

		A_{450}		
Metal	Concentration	Active catalase	Boiled catalase	Difference
Mn^{2+}	10^{-4} *M*	0.722	0.536	0.186
	10^{-5} *M*	0.215	0.231	−0.016
Cu^{2+}	10^{-4} *M*	1.727	1.437	0.290
	10^{-5} *M*	0.829	0.643	0.186
None		0.096	0.106	−0.010

[a] Catalase source was Worthington CTS beef liver catalase, diluted fivefold with water. Metal ion concentrations are those in the final mixture.

reaction rate is greatly accelerated by the presence of hemoglobin (11) or catalase. It is not clear why Morgan *et al.* (16), using a soluble liver fraction from poikilothermic vertebrates, found that 10^{-3} *M* Cu^{2+} or Mn^{2+} showed no effect on the reaction.

Summary. The introduction of the acatalasemic gene locus, Cs[b], which presumably affects only catalase biosynthesis, not only reduces the catalase activity of blood and liver in two inbred strains of mice, but comparably reduces the activity of cinnabarinate synthase. This enzyme converts the carcinogen hydroxyanthranilic acid to another carcinogen, cinnabarinic acid. The injection of aminotriazole, which inhibits the catalase activity of the liver but not of the blood, similarly inhibits the cinnabarinate synthase activity of the liver and not of the blood. The proteins responsible for the two enzyme activities therefore appear identical, confirming the recent suggestion of Savage and Prinz. The cinnabarinate synthase reaction can also be carried out by Cu^{2+} or Mn^{2+} in absence of any enzyme, but addition of a source of catalase or hemoglobin accelerates the reaction. Catalase or hemoglobin, in the absence of Cu^{2+} or Mn^{2+}, are incapable of carrying out the oxidative dimerization of hydroxyanthranilic acid.

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Received January 30, 1978. P.S.E.B.M. 1978, Vol. 158.