CATALASE ACTIVATES CEREBRAL GUANYLATE CYCLASE IN THE PRESENCE OF SODIUM AZIDE

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SUMMARY

Mouse cerebral guanylate cyclase was markedly activated by sodium azide in the presence of a macromolecular guanylate cyclase activating factor (GAF), which was found to be rich in mitochondrial fractions from liver and kidney. Catalase from the beef liver also activates cerebral guanylate cyclase in the presence of NaN_3. The ability of both GAF and catalase to activate cerebral guanylate cyclase was abolished by heating, but resistant to trypsin digestion. NaN_3 was replaced by hydroxylamine but not by CN^- or F^-. Catalase resembles GAF in several biochemical parameters tested and may play an important role for regulating guanylate cyclase activity.

INTRODUCTION

Kimura et al. (1) reported that NaN_3 activates significantly both soluble and particulate-bound guanylate cyclase (GC) in the rat liver, and this activation depends on a macromolecule in liver supernatant. We found that GC in the homogenate of mouse cerebral cortex is not significantly activated by NaN_3, but it is fully activated when a small amount of liver homogenate is added to the assay system. This indicates the existence of a macromolecular guanylate cyclase activating factor (GAF) in liver homogenate. In this paper we describe some properties of GAF, which was extracted and partially purified from mitochondrial fractions of the liver and kidney. Since it was found that catalase also activates cerebral GC in the presence of NaN_3 or NH_2OH, some comparisons were made to see whether or not GAF and catalase have identical or similar properties concerning the activation of GC.

MATERIALS AND METHODS

Porcine kidney was obtained from a local slaughter house. Cerebral cortices of male mouse (STD-dd/Y) were homogenized with 20 volumes of 0.25 M su-

crose using a glass homogenizer with a Teflon pestle. All procedures were done at 4°C.

Partial Purification of GAF - Mouse liver (8gr), perfused with 0.25 M sucrose, was homogenized with 10 volumes of 0.25 M sucrose using a Teflon homogenizer. Porcine kidney cortices (200gr) were homogenized with 5 volumes of 0.25 M sucrose in a Waring Blender for 1 min. The homogenate was filtered through three layers of gauze and centrifuged at 600 × g for 10 min. The supernatant obtained was further centrifuged at 15,000 × g for 5 min and the resulting pellet (mitochondrial fraction) was suspended in 20 mM Tris HCl buffer, pH 7.5. This particulate suspension was frozen in dry ice-acetone and thawed in water bath at 30°C. After repeating the freezing-thawing procedures 3 times, the suspension was centrifuged at 105,000 × g for 60 min. Ammonium sulfate was added to the supernatant fraction to yield a 35-65 % saturated fraction. The resulted precipitate was dissolved in 20 mM Tris HCl buffer, pH 7.5 and dialyzed against the same buffer. One unit of GAF activity is arbitrarily defined as the amount which is required to give 50 % stimulation of cerebral GC in the presence of sodium azide.

Guanylate Cyclase Assay - GC activity was assayed in 20 µl of the reaction mixture containing 50 mM Tris HCl buffer (pH 7.7), 4 mM $MnCl_2$, 0.1 mM [^{3}H] GTP (diluted to 500 Ci/mol, Radiochemical centre, Amersham), 0.5 mM isobutylmethylxanthine, 10 mM theophylline, 3 mM guanosine 3':5'-monophosphate (cGMP), 15 mM creatine phosphate, 20 µg of creatine kinase and the enzyme sample (10-40 µg of protein). Enzyme activity was determined by incubating in the presence or absence of 2 mM NaN_3 for 10 min at 37°C and terminated by the addition of 5 µl of 2 M acetic acid and boiling for 2 min. cGMP was isolated by thin layer chromatography as previously described (2). Radioactive cGMP was produced linearly for at least 15 min under the standard assay conditions. The molecular weight and sedimentation coefficient of the GAF were estimated by the method of Martin and Ames (3), using a continuous sucrose density gradient (5-20 %) centrifugation. Catalase (11.6s, molecular weight: 232,000) (4) and yeast alcohol dehydrogenase (6.7s, molecular weight: 150,000) (5) activities were assayed as previously described. Beef liver catalase and yeast alcohol dehydrogenase were purchased from Sigma Chemical Co. Protein concentration was determined by the method of Lowry et al. (6).

RESULTS

When GAF activity was examined in various tissue homogenates, only liver and kidney were found to contain GAF activity. The tissue homogenates from other organs such as heart, erythrocyte and lung virtually inhibited the cerebral GC activity. The activity of GAF in mouse liver was twice as high as kidney homogenate (Fig. 1). When subcellular locarization of the GAF was studied in mouse liver homogenate, it was found that approximately 50 % of the GAF activity is associated with mitochondrial fraction, in which little activity of GC is found (Table I). The GAF activity in porcine kidney was also found to be the highest in mitochondrial fraction. The supernatant of liver homogenate showed a substantial activity of GC, but a low activity of GAF. The GAF was extracted from mitochondrial fraction and partially pu-

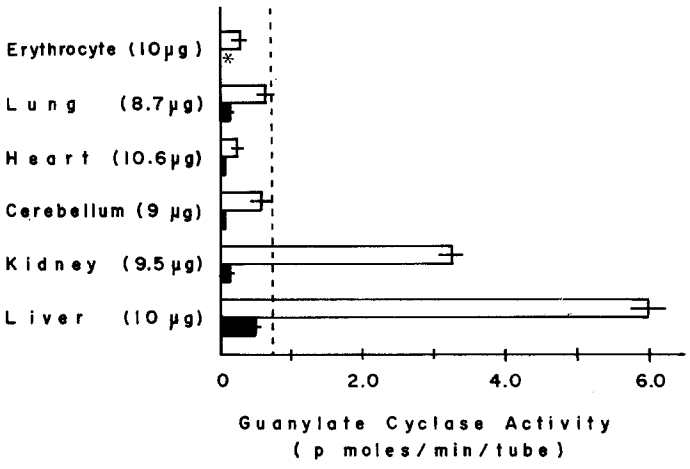

Fig. 1. Distribution of GAF activity in various tissue homogenate of mouse: Each tissue homogenate (protein content was indicated respectively in parentheses) was mixed with cerebral homogenate (approximately 30 μg protein) and then GC activity in the mixture was measured (▭). The closed column (▬) and the dotted line represent the GC activity of each tissue homogenate in the absence of cerebral homogenate, and the GC activity of cerebral homogenate without adding any other tissue homogenates, respectively. All GC activities presented in this figure were assayed in the presence of 2 mM NaN_3. The maximal activity of cerebral GC obtained was 277±24 pmoles cGMP synthesized/mg prot/min. Results are expressed in pmoles cGMP synthesized/min/tube ±S.D. *Erythrocyte had no appreciable GC activity.

Table I

Distribution of activities of GAF and GC in fractions from mouse liver homogenate

Fraction	GAF Activity (% of Homogenate)	GC Activity (% of Homogenate)
Nuclear	21	10
Mitochondrial	48	5
Microsomal	15	2
Supernatant	5	80

GAF and GC activities were assayed as described under Materials and Methods. GAF and GC activities in liver homogenate were 135 ± 11 units/mg protein and 110 ± 5 pmoles cGMP synthesized/mg prot/min, respectively. Data shown are the averages of four separate determinations. Subcellular fractions of the liver homogenate were prepared with the method of Hogenboom (7).

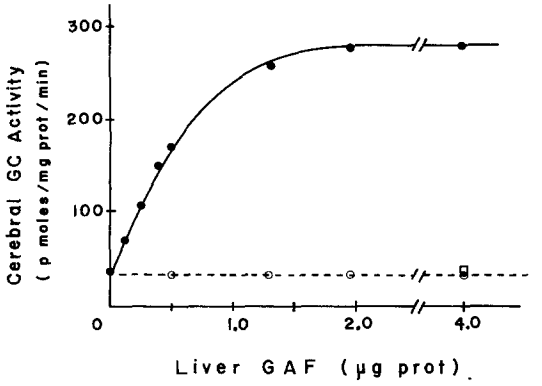

Fig. 2. Activation of cerebral GC by partially purified GAF from mouse liver: Various amounts of the partially purified liver GAF were mixed with cerebral homogenate (approximately 30 µg protein) and then GC activity in the mixture was measured in the presence (●——●) or absence (○- - -○) of 2 mM NaN_3. The open square (□) indicates cerebral GC activity measured in the presence of heat treated-GAF (90°C, 5 min) and 2 mM NaN_3.

rified (see Materials and Methods). The GAF activities in the homogenates of mouse liver (139 units/mg protein) and kidney cortex (6.8 units/mg protein) were purified respectively to 2,500 units/mg protein and 432 units/mg protein. These partially purified GAF retained less than 0.5 % of GC activity found in the original homogenates. The half-maximal activation of cerebral GC was achieved by the addition of approximately 0.4 µg protein of the partially purified liver GAF in the presence of 2 mM NaN_3 (Fig. 2). In the partially purified GAF from the porcine kidney, half-maximal activation of the cerebral GC was produced by approximately 3 µg of protein. The GAF was resistant to digestion by trypsin (100γ/ml, 30 min, 25°C), but inactivated by heating (at 90°C for 3 min). When the partially purified GAF was analyzed on a continuous density gradient centrifugation using 5-20 % sucrose, it was found that the GAF has a sedimentation coefficient of 11.6s (about 240,000 daltons), which corresponds to that of catalase. When the recrystallized beef liver catalase was centrifuged with the partially purified liver GAF, GAF activity was recovered in the same fractions as that of catalase and these fractions exhibited much more GAF activity than fractions recovered

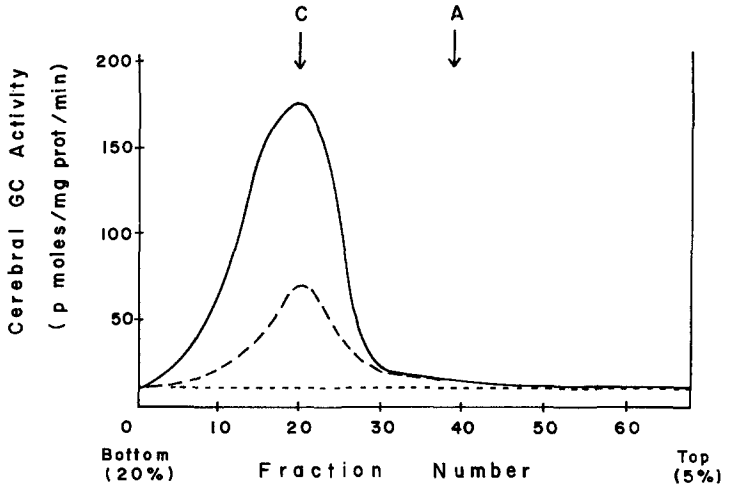

Fig. 3. Sucrose density gradient profille of the liver GAF:
The partially purified liver GAF was divided into two 100-µl fractions with approximately 0.2 mg protein in each. Beef liver catalase (80 µg protein) was added to one and nothing to the other. Yeast alcohol dehydrogenase was added to each. Then in separate tubes each sample (0.15 ml) was layered on the top of 4.5 ml of a continuous sucrose gradient (5-20 %, in 20 mM Tris HCl buffer, pH 7.5) and centrifuged at 35,000 rpm in Hitachi SW 39 rotor for 14 hrs at 4°C. Contents of the tube were harvested from the bottom into 68 equal fractions. One µl of each fraction was mixed with 10 µl of cerebral homogenate (approximately 30 µg protein) and GC activity was measured in the presence of 2 mM NaN_3. The results show GAF activity in the presence (——) or absence (— —) of catalase. The dotted line (------) represents GAF activity measured in the absence of NaN_3. The fractions having the maximal catalase (C) and yeast alcohol dehydrogenase (A) activities are indicated respectively by arrows.

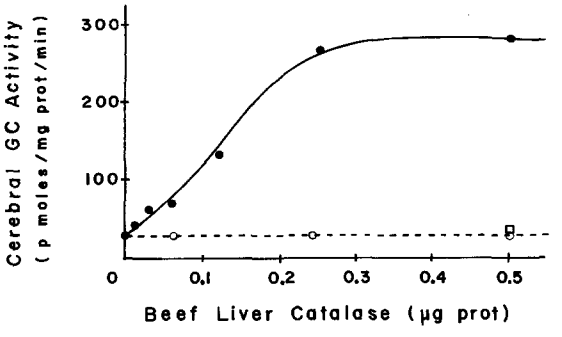

Fig. 4. Activation of cerebral GC by catalase:
Various protein concentrations of the recrystallized catalase from beef liver were mixed with cerebral homogenate (about 30 µg protein) and then GC activity was measured in the presence (●——●) or absence (○- - -○) of 2 mM NaN_3. The open squar (□) indicates cerebral GC activity in the presence of heat treated-catalase (90°C, 5 min) and 2 mM NaN_3.

from a sample without containing catalase (Fig. 3). This suggests that catalase itself may activate cerebral GC in the presence of NaN_3. In fact the recrystallized catalase from the beef liver significantly activated cerebral GC. Half-maximal activation of cerebral GC was achieved at a concentration of approximately 0.15 μg protein of catalase in the presence of 2 mM NaN_3 (Fig. 4). The ability of catalase to activate cerebral GC was abolished by heating, but resistant to trypsin digestion. In the absence of NaN_3, no activation of cerebral GC was produced by the addition of catalase. It was confirmed that catalase itself has no capacity to produce cyclic GMP from GTP. NH_2OH could be substituted for NaN_3 in the activation of GC, but not by CN^-, F^-, Fe^{3+} and ascorbate + Cu^{2+} (Table II). The hemolysate of erythrocytes suppressed the effect of catalase or GAF on GC activation in the presence of NaN_3.

Table II

Effect of various agents on the ability of GAF and catalase to activate cerebral GC

Addition	Cerebral GC Activity p moles cGMP synthesized/mg prot/min ± S.D.	
	Liver GAF	Catalase
None	21± 1	22± 1
NaN_3, 2 mM	260±24	255±27
NH_2OH, 2 mM	156±15	182±19
NaCN, 2 mM	20± 1	18± 1
NaF, 20 mM	19± 1	18± 1
Ascorbate, 1 mM + $CuSO_4$, 5 μM*	18± 1	18± 1
$FeCl_3$, 5 μM	20± 1	20± 2
Erythrocyte hemolysate, 10 μg	18± 1	18± 1
Erythrocyte hemolysate, 10 μg + NaN_3, 2 mM	18± 1	18± 1

Cerebral GC activity was assayed in the presence of the partially purified liver GAF (4 μg of protein) or catalase (0.5 μg of protein). Mouse erythrocytes were washed twice with 0.9 % of NaCl and then lysed in 10 mM Tris HCl buffer, pH 7.5.
* Liver GAF or catalase was preincubated with ascorbate and $CuSO_4$ for 20 min at 37°C before adding to the assay system for GC activity. Each value in this table represents the mean ± S.D. obtained from four separate experiments.

DISCUSSION

GAF activity was found only in the liver and kidney homogenates, but not in other tissue homogenates examined. Erythrocyte which contains catalase did not activate cerebral GC, but rather inhibited it. Kimura et al.(1) reported that soluble fraction from the heart prevents the activation of liver-soluble GC by NaN_3 and suggested the presence of an inhibitor of the NaN_3 response. We found the hemolysate of erythrocytes also prevents GC activation by GAF or catalase in the presence of NaN_3. These results suggest that erythrocyte may also have an inhibitor of the GC activation by GAF or catalase in the presence of NaN_3. Mitochondrial fractions from the kidney and liver showed the highest GAF activity and approximately 50 % of the activity was solubilized in a hypotonic medium by repeated freezing and thawing procedures. Catalase in the liver is known to be stored in microbodies involved in mitochondrial fractions (8) and our results also suggest that the GAF may have a similar mitochondrial localization. The partially purified GAF from the mouse liver and porcine kidney resemble catalase in molecular size (about 240,000 daltons), trypsin-resistance, heat-lability and the ability of activating GC in the presence of NaN_3 or NH_2OH. NaN_3, NH_2OH, CN^-, F^-, Fe^{3+} and ascorbate + Cu^{2+} are all known as inhibitors of catalase (4,9,10). The fact that only NaN_3 and NH_2OH activate GC in the presence of GAF or catalase, but CN^-, F^-, Fe^{3+} and ascorbate + Cu^{2+} have no such effects, suggesting that the capacity of NaN_3 and NH_2OH for activating GC may not be directly related to the inhibitory action of these agents on catalase activity. NaN_3 and NH_2OH are known to bind to hemins of catalase molecule and reduce Fe^{3+} to Fe^{2+} (10). Although molecular mechanisms underlying the catalase induced-activation of GC in the presence of NaN_3 or NH_2OH remain to be elucidated, we wish to propose a working hypothesis that the molecular conformational changes of catalase which is induced by NaN_3 or NH_2OH may participate in the activation of GC.

REFERENCES

1. Kimura, H., Mittal, C.K., and Murad, F. (1975) J. Biol. Chem, 250, 8016-8022.
2. Keirns, J.J., Wheeler, M.A., and Bitensky, M.W. (1974) Anal. Biochem, 61 336-348.
3. Martin, R.G., and Ames, B.N. (1961) J. Biol. Chem, 236, 1372-1379.
4. Chance, B., and Maehly, A.C. (1955) Methods in Enzymol, 2, 764-768.
5. Racker, E. (1955) Methods in Enzymol, 1, 500-503.
6. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem, 193, 265-275
7. Hogeboom, G.H. (1955) Methods in Enzymol, 1, 16-19.
8. Baudhuin, P., Beaufay, H., and DeDuve, C. (1965) J. Cell Biol, 26, 219-243.
9. Orr, C.W.M. (1970) Methods in Enzymol, 18A, 59-62.
10. Theorell, H., and Ehrenberg, A. (1952) Arch. Biochem. Biophys, 41, 442-474.