Formation of Adenosine 3'-Monophosphate in Rat Liver Mitochondria

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An unknown adenine-related compound (UKC) in rat liver mitochondria was characterized. Based on the sensitivity to periodate oxidation, nuclease P₁ digestion, property of fluorescence derivatization, elution behavior on different separation modes of HPLC columns and the mass spectrum of purified UKC, the UKC was identified as adenosine 3'-monophosphate (3'-AMP), an intracellular P-site inhibitor of adenylate cyclase. 3'-AMP may be enzymatically produced from RNA in rat liver mitochondria in temperature- and time-dependent manners. A partial characterization of 3'-AMP forming enzyme is included.

Key words adenosine 3'-monophosphate; mitochondria; rat; liver; chloroacetaldehyde

Almost all intracellular ATP are known to be generated in mitochondria¹⁾ and about one-third of the cellular adenine nucleotides are located in this organelle.²⁾ Some nucleotides are generated from the inherent DNA and various RNAs³⁾ degraded by DNAase^{4,5)} and RNAase,^{4–6)} respectively. Therefore, intramitochondrial adenine nucleotide levels may be regulated not only by energy generating ability but also by polynucleotide-degrading activity under both physiological and pathophysiological conditions.

During the determination of rat liver mitochondrial adenine nucleotides such as ATP, adenosine 5'-diphosphate (ADP) and adenosine 5'-monophosphate (AMP) using an Asahipak GS-320H of a size-exclusion HPLC column with a fluorescent-reaction,^{7,8)} an unknown chloroacetaldehyde-reactive compound (UKC) was found to be eluted between AMP and cAMP. This fact promoted us to clarify what the UKC was and what factors affected this UKC formation.

In the present study we report that the UKC was enzymatically produced in the isolated rat liver mitochondrial fraction and that the UKC was identified as 3'-AMP, an intracellular P-site inhibitor of adenylate cyclase.^{9,10)}

MATERIALS AND METHODS

Materials Asahipak GS-320H (250×7.6 mm i.d.) for size exclusion was purchased from Asahi Chemical Industry (Kanagawa, Japan). An HPLC column (250×4.6 mm i.d.) of Chromatorex ODS (DU0005MTP, 5 μm) was kindly supplied by Fuji Silysia Chemical, Ltd. (Aichi, Japan). Chloroacetaldehyde was obtained from Wako Pure Chemicals Co. (Japan). 3'-AMP, 2'-deoxyadenosine (dAdo), 2'-deoxy-5'-AMP (dAMP) and nuclease P₁ from *Penicillium citrinum* were from Yamasa Shouyu Co. (Japan). Adenosine 2',3'-cyclic monophosphate (2',3'-cAMP), 2-deoxy-3'-AMP (3'-dAMP), adenosine 2'-monophosphate (2'-AMP), cytidine 3'-monophosphate (3'-CMP) and cytidine 5'-monophosphate (5'-CMP) were from Sigma Chemical Co. (U.S.A.). Other chemicals of reagent grade were commercially obtained.

Preparation of Crude Mitochondria from Rat Liver Mitochondria were isolated from the liver of a male albino Wistar rat weighing ca. 250 g by a modification of the method described by Schneider.¹¹⁾ Mitochondria thus obtained were washed twice with 0.1 M phosphate buffer (pH 7.0), then suspended in the same buffer to make ca. 2.5 mg protein per ml. Protein was determined by the method of

Lowry *et al.*¹²⁾ using bovine serum albumin as a standard.

Determination of Mitochondrial Adenine Compounds by HPLC One volume of crude mitochondrial suspension obtained above was added to one vol. of 2 M perchloric acid. The acid-soluble compounds were treated with chloroacetaldehyde for fluorescence derivatization as described previously.^{7,8)} The derivatized compounds were analyzed by HPLC using a column of Asahipak GS-320H maintained at 40 °C. The mobile phase consisted of 0.1 M citric acid–0.2 M disodium hydrogen phosphate (pH 5.0) and methanol (9 : 1, v/v). The flow-rate was 1 ml/min. The eluate was detected by an Intelligent spectrofluorometer 820-FP (Jasco, Japan).

The non-derivatized acid-soluble compounds were analyzed by HPLC using a column of Chromatorex ODS (DU0005MTP) maintained at 40 °C. The mobile phase was 0.1 M sodium acetate–acetic acid (pH 4.5). The flow-rate was 1.0 ml/min, and the eluate was monitored at 254 nm by an Intelligent UV/VIS Detector 875-UV (Jasco).

Preparation of Mitochondrial Extract Mitochondria were suspended in 0.1 M phosphate buffer (pH 7.0) and incubated at 37 °C for 4 h. Acid-soluble compounds containing UKC in the mitochondria were extracted with 2 M perchloric acid according to the procedure described by us,^{7,8)} except for derivatization with chloroacetaldehyde.

Periodate Oxidation Periodate oxidation of UKC and authentic nucleic acids was done according to the method described by Garrett and Santi.¹³⁾

Purification of UKC The UKC in acid-soluble mitochondrial extract obtained above was purified using an HPLC column packed with a Hitachi gel No. 3013-N anion exchanger resin and then an HPLC column of Chromatorex ODS. Mass spectrum of the purified UKC was taken with a JEOL JMS-DX300 mass spectrometer.

RESULTS

The adenine compounds derivatized with chloroacetaldehyde were well separated on an Asahipak GS-320H by an isocratic elution, and eluted from the column in the order of ATP, ADP, AMP, cAMP, Ado and dAdo. An unknown chloroacetaldehyde-reactive compound (UKC) eluted between AMP and cAMP was found (Fig. 1). Under these conditions, authentic 3'-AMP, 3'-dAMP and dAMP were eluted between AMP and cAMP, and 2'-AMP was not separated from AMP on the column.

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Fig. 1. Chromatogram of Authentic Adenine Compounds and UKC (3'-AMP) on a Column of Asahipak GS-320H

Ten microliters of 4 μ M adenine compounds in 0.1 M Hepes buffer (pH 7.0) and a pooled eluate corresponding to the retention time of the UKC on a column of Chromatorex ODS were injected. The relative fluorescence intensity (RFI) was drawn.

Table 1. Effect of Incubation Time on Adenine Compound Levels from Rat Liver Mitochondria

Time (h)	nmol/mg Protein				cm ^a /mg Protein
	ATP	ADP	AMP	Total ^b	
0	7.85 $\pm$ 0.12	0.46 $\pm$ 0.02	4.25 $\pm$ 0.06	12.56 $\pm$ 0.05	ND
3	0.09 $\pm$ 0.00	1.03 $\pm$ 0.07	0.48 $\pm$ 0.02	1.60 $\pm$ 0.09	4.6 $\pm$ 0.08
6	0.08 $\pm$ 0.00	0.93 $\pm$ 0.03	0.79 $\pm$ 0.02	1.80 $\pm$ 0.05	12.2 $\pm$ 1.00
24	0.17 $\pm$ 0.02	0.15 $\pm$ 0.01	0.91 $\pm$ 0.03	1.23 $\pm$ 0.05	104.1 $\pm$ 7.80

ND: not detectable. ^a peak height, ^b ATP+ADP+AMP, ^c unknown compound. The mitochondrial fraction in 0.1 M phosphate buffer (pH 7.0) was incubated at 30 °C for the indicated h. All values are presented as means $\pm$ S.E. of triplicates.

As shown in Table 1, total adenine compound levels in the isolated mitochondria incubated for 3 to 24 h were about 10–14% of that in freshly prepared mitochondria in a phosphate buffer (pH 7.0). The height of chloroacetaldehyde-reactive UKC was increased as a function of the incubation time when the isolated mitochondria were incubated in the phosphate buffer. The production of UKC was augmented in a temperature-dependent manner (data not shown). After incubation of the mitochondria in the phosphate buffer at 37 °C for 1 h, the UKC level in the mitochondria obtained by centrifugation was *ca.* 8–10 fold higher than that in the resultant supernatant, suggesting that the UKC formation occurred in the mitochondria.

The chloroacetaldehyde-reactive UKC was not separated well from AMP on an HPLC column packed with a Hitachi gel No. 3013-N anion exchanger resin, which is used for a routine determination of adenine compounds,^{7,8)} indicating that the UKC may be an adenine monophosphate-related compound (data not shown). The UKC, as well as the authentic 3'-AMP, 2'-AMP, dAMP, 3'-dAMP, cAMP and dAdo, were not degraded by periodate oxidation. As shown in Fig. 2-B, the UKC disappeared along with the appearance of Ado on the HPLC chromatogram. Under the nuclease P₁ digestion,¹⁴⁾ dAdo and Ado were released from 3'-dAMP (Fig. 2-F) and 3'-AMP (Fig. 2-D), respectively, but dAMP and 2'-AMP were not hydrolyzed by the enzyme under the experimental condition (data not shown).

The retention time of the UKC was exactly the same as that of authentic 3'-AMP on a column of Chromatorex ODS. In addition, the UKC was co-eluted with authentic 3'-AMP

Fig. 2. Chromatograms of Rat Liver Mitochondrial Extract Treated with or without Nuclease P₁ on a Column of Asahipak GS-320H

Fifty microliters of the mitochondrial extract (Mit) and 20 μ M of 3'-AMP or 3'-dAMP were treated with 5 units of nuclease P₁ (NP₁) dissolved in 0.1 M phosphate buffer (pH 7.4) containing 2 mM ZnCl₂. A) Mit, B) Mit treated with NP₁, C) mixture of Mit and 3'-AMP, D) mixture of Mit and 3'-AMP treated with NP₁, E) mixture of Mit and 3'-dAMP, F) mixture of Mit and 3'-dAMP treated with NP₁.

on a column of Asahipak GS-320H when a mixture of the UKC and 3'-AMP was applied on the column. Under the elution conditions, 2'-AMP, dAMP, 3'-dAMP, 2',3'-cAMP, 5'-CMP and 3'-CMP, which are known to be derivatized with chloroacetaldehyde to form an ethenocytidine compound,^{15,16)} were well separated from the UKC and 3'-AMP on the reversed phase HPLC column. Inosine and hypoxanthine, which are metabolites of AMP in rat mitochondria,¹⁷⁾ lacked their reactivities with the aldehyde.

As shown in Fig. 3, the positive-ion and negative-ion FAB mass spectra of the purified UKC showed molecular ion peaks at *m/z* 348 and 346, respectively, indicating that the molecular weight of the UKC was 347, which is identical with that of authentic 3'-AMP.

DISCUSSION

The viability (or freshness) of various tissues or cells depends mainly on the ATP producing ability of their mitochondria. The total amount of rat liver mitochondrial adenine compounds determined by a fluorescent-HPLC was in good agreement with the values of 10–13 nmol/mg protein as reported by others.^{2,18–20)} Therefore, an HPLC column of Asahipak GS-320H for size exclusion is available to estimate the viability of biological samples.

The UKC eluted between AMP and cAMP from an Asahipak GS-320H column was identified as 3'-AMP on the basis of its periodate oxidation behavior, degradation profile by nuclease P₁ (Fig. 2), and chromatographic profile of the UKC, 3'-AMP on different separation modes of HPLC columns for anion-exchange, reversed-phase and size-exclusion (Fig. 1) and mass spectrum of the purified UKC (Fig. 3). The formation of UKC (3'-AMP) was thought to be due to an enzymatic reaction in the mitochondrial fraction, and the substrate for releasing 3'-AMP might be mitochondrial RNAs because the UKC was produced in temperature- and

Fig. 3. Positive-ion (A) and Negative-ion (B) FAB Mass Spectra of the Purified UKC
Matrix: glycerin.

time-dependent manners (Table 1). Two kinds of RNAase classified as exonuclease^{21,22)} and endonuclease^{23,24)} have been reported in rat liver microsomes. The purified exonuclease, which required Mg^{2+} for activation, catalyzed the release of AMP from the substrate poly(A).²²⁾ The endonuclease produces oligonucleotides from poly(A), and no AMP or 3'-AMP are released from poly(A) by this enzyme.²⁴⁾ Mammalian mitochondrial ribonuclease is known to be a site-specific endonuclease which requires an RNA component for its activity.⁶⁾ In the present experiment, the formation of UKC (3'-AMP) was only found when isolated mitochondria were incubated in 0.1 M phosphate buffer (Table 1) or 0.1 M Tris-HCl (pH 7.4) containing 0.1 M EDTA, but no UKC was detected in mitochondria incubated in a phosphate buffer containing Mg^{2+} (data not shown). In addition, no chloroacetaldehyde reactive oligonucleotides were detected on the chromatograms obtained from the Asahipak GS-320H column (Fig. 2). These results suggest that 3'-AMP forming enzyme(s) in rat mitochondria might be an exonuclease and do not depend on Mg^{2+} for activation. Poly(A) polymerase, which synthesizes poly(A) from ATP substrate, can also hydrolyze poly(A).²⁵⁾ The enzyme is known to be localized in mitochondria,²⁶⁾ and to be 3'-exonuclease, which requires Mg^{2+} for activation and predominantly releases AMP but not 3'-AMP.²⁵⁾ To our knowledge, this is the first paper describing that an interesting characteristic of exonuclease, 3'-AMP forming RNAase, exists in rat liver mitochondria.

It has been shown that membrane-bound adenylate cyclase can be regulated by a number of extracellular and intracellular signals. 3'-AMP is known to be classified as a P-site inhibitor of adenylate cyclase.^{9,10)} Bushfield *et al.*¹⁰⁾ reported that rat liver contained 3'-AMP and speculated that a 3'-AMP forming enzyme might be involved in RNA metabolism. If 3'-AMP formation occurs in cytosol, the nucleotide may play significant roles in the regulation of adenylate cyclase activity. Therefore, the formation and accumulation of 3'-AMP in mitochondria may affect cell functions via the inhibition of adenylate cyclase. The physiological and pathophysiological significance of 3'-AMP formation in mitochondria remains to be elucidated.

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REFERENCES

- 1) Erecinska M., Wilson D.H., *J. Memb. Biol.*, **70**, 1—14 (1982).
- 2) Aprille J. R., Austin J., *Arch. Biochem. Biophys.*, **212**, 689—699 (1981).
- 3) Anderson S., Bankier A. T., Barrell B. G., de Bruijn M. H. L., Coulson A. R., Drouin J., Eperon I. C., Nierlich D. P., Roe B. A., Sanger F., Schreier P. H., Smith A. J. H., Staden R., Younget I. G., *Nature* (London), **290**, 457—465 (1981).
- 4) Curtis P. J., Burdon M. G., Smellie R. M. S., *Biochem. J.*, **98**, 813—817 (1966).
- 5) Curtis P. J., Smellie R. M. S., *Biochem. J.*, **98**, 818—825 (1966).
- 6) Chang D. D., Clayton D. A., *Science*, **235**, 1178—1184 (1987).
- 7) Fujimori H., Yamauchi M., Pan-Hou H., *Chem. Express*, **6**, 715—718 (1991).
- 8) Fujimori H., Sasaki T., Hibi K., Senda M., Yoshioka M., *J. Chromatogr.*, **528**, 305—314 (1990).
- 9) Johnson R. A., Yeung S. H., Stubner D., Bushfield M., Shoshani I., *Mol. Pharmacol.*, **35**, 681—688 (1989).
- 10) Bushfield M., Shoshani I., Johnson R. A., *Mol. Pharmacol.*, **38**, 848—853 (1990).
- 11) Schneider W. C., *J. Biol. Chem.*, **176**, 259—266 (1948).
- 12) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
- 13) Garrett C., Santi D. V., *Anal. Biochem.*, **99**, 268—273 (1979).
- 14) Fujimoto M., Kuninaka A., Yoshino H., *Agr. Biol. Chem.*, **38**, 1555—1561 (1974).
- 15) Kochetkov N. K., Shibaev V. N., Kost A. A., *Tetrahedron Lett.*, **22**, 1993—1996 (1971).
- 16) Barrio J. R., Secrist III J. A., Leonard N. J., *Biochem. Biophys. Res. Commun.*, **46**, 597—604 (1972).
- 17) Ziegler M., Dubiel W., Pimenov A. M., Tikhonov Y. V., Toguzov R. T., Henke W., Gerber G., *Biomed. Biochim. Acta*, **48**, 57—61 (1989).
- 18) Pollak J. K., Sutton R., Klingenberg M., *Biochem. Biophys. Res. Commun.*, **80**, 193—198 (1978).
- 19) Asimakis G. K., Aprille J. R., *Arch. Biochem. Biophys.*, **203**, 307—316 (1980).
- 20) Tager J. M., Wanders R. J. A., Groen A. K., Kunz W., Bohnensack R., Kuster U., Letko G., Bohme G., Duszynski J., Wojtczak L., *FEBS Lett.*, **151**, 1—9 (1983).
- 21) Kumagai H., Igarashi K., Tanaka K., Nakao H., Hirose S., *Biochim. Biophys. Acta*, **566**, 192—199 (1979).
- 22) Kumagai H., Nakamura M., Ozaki N., Igarashi K., Hirose S., *Biochim.*

- Biophys. Acta*, **827**, 431—438 (1985).
- 23) Kumagai H., Igarashi K., Takayama T., Watanabe K., Sugimoto K., Hirose S., *Biochim. Biophys. Acta*, **608**, 324—331 (1980).
- 24) Kumagai H., Abiko T., Ono C., Marumo Y., Enomoto S., Igarashi K., Hirose S., *Biochim. Biophys. Acta*, **827**, 424—430 (1985).
- 25) Abraham A. K., Jacob S. T., *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 2085—2087 (1978).
- 26) Rose K. M., Morris H. P., Jacob S. T., *Biochemistry*, **14**, 1025—1032 (1975).