A Specific and Rapid Method for Determination of Adenosine 3'-Monophosphate (3'-AMP) Content and 3'-AMP Forming Enzyme Activity in Rat Liver Mitochondria, Using Reversed-Phase HPLC with Fluorescence Detection

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Received July 6, 1998; accepted September 7, 1998

To study the physiological significance of adenosine 3'-monophosphate (3'-AMP), an intracellular P-site inhibitor of adenylyl cyclase, in rat liver mitochondria, a specific, rapid and reliable assay method for determination of 3'-AMP and the activity of its forming enzyme is required. 3'-AMP in rat liver was determined to be ca. 23±7 nmol/g wet weight, but no 2-deoxy-3'-AMP, another P-site inhibitor of adenylyl cyclase, was detected, even when using a reversed-phase HPLC column with a fluorescent-reaction, as established in this study. By using the optimized assay method developed here, 3'-AMP forming enzyme activity in rat crude mitochondrial extract was found to be enhanced by EDTA and inhibited by p-chloromercuribenzoate. The optimum pH was ca. 5.8 and no divalent cation was required for activity. From these results, 3'-AMP forming enzyme(s) in rat liver mitochondria could be classified as acid exoribonuclease, which mainly existed in an active form. The results obtained in this study will help to gain more insight into the physiological roles of 3'-AMP in living systems.

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

Adenylyl cyclase is known to be regulated by various extracellular and intracellular signals. Intracellular adenosine 3'-monophosphate (3'-AMP), one of the degradation products of various RNAs, has been pharmacologically classified as a P-site inhibitor of adenylyl cyclase.1-3 However, the physiological and pathophysiological significance of 3'-AMP still remains to be elucidated.

Recently, Fujimori and Pan-hou4 suggested that 3'-AMP detected in rat crude liver mitochondria may be enzymatically produced from mitochondrial RNA by putative 3'-AMP forming enzyme(s), one of the ribonucleases (RNase). It is known that rat mitochondria possess several kinds of RNases5,6 which catalyze the production of adenosine 5'-monophosphate (AMP) or oligoribonucleotides, but not 3'-AMP. Therefore, it is of interest to clarify whether 3'-AMP forming enzymes exist in rat liver mitochondria.

Most RNase activity is determined by measuring the absorbance or radioactivity of released mononucleotides and oligonucleotides from substrate yeast RNA or poly(A) that are labelled without5,6 or with 3H7,8 or 32P9,10. These spectrophotometric and radiochemical methods can not directly identify what kinds of nucleotides are released from the substrates used. Several methods for determination of 3'-AMP have been reported11-13. However, the developed methods are less specific for detection of 3'-AMP and are difficult to perform. To characterize the 3'-AMP forming enzyme in biological samples, a convenient and specific method is required.

In the present report we developed a rapid and reliable method for determining 3'-AMP forming enzymatic activity by using a reversed-phase HPLC column with fluorescence detection. By using the described method, we demonstrate that 3'-AMP forming enzyme(s), which can be classified as exoribonuclease, exist in rat liver mitochondria.

MATERIALS AND METHODS

Materials An HPLC column (150×4.6 mm i.d.) of Chromatorex ODS (DU0005MT, 5 μm) was kindly supplied by Fuji Sylvania Chemical, Ltd. (Aichi, Japan). Chloroacetaldehyde was purchased from Wako Pure Chemicals Co. (Japan). 3'-AMP and poly(A) were from Yamasa Shouyu Co. (Japan). 2'-Deoxy-3'-AMP (3'-dAMP) and adenosine 2'-monophosphate (2'-AMP) were from Sigma Chemical Co. (U.S.A.). Other chemicals of reagent grade were commercially obtained.

Determination of Adenine Compounds by HPLC Extraction of adenine compounds in rat liver with perchloric acid was performed according to the method described by us.4,13 The acid-soluble compounds were derivatized with chloroacetaldehyde for fluorescence detection. The derivatized compounds were analyzed by a reversed-phase HPLC using a column of Chromatorex ODS maintained at 45 °C. The mobile phase consisted of 0.01-0.1 μ mol acetate buffer (pH 4.2-5.0) and methanol (final % of 1-6). The flow-rate was 1 ml/min. The eluate was detected by an Intelligent spectrophotometer 820-FP (Jasco, Japan).4,13

Preparation of Crude Mitochondrial Extract from Rat Liver Mitochondria were isolated from the livers of male albino Wistar rats weighing ca. 400-450 g by a modification of the method described by Schneider.14 Mitochondria were suspended in 0.1 M phosphate buffer (pH 7.0) to give ca. 2.5 mg protein per ml.15 The mitochondria obtained were frozen and thawed twice. The treated mitochondria were then centrifuged at 14000×g for 20 min to separate mitochondrial pellet and supernatant. The supernatant was kept at -80 °C until use and used as a crude enzyme solution. Protein was determined by the method of Lowry et al.16 using bovine serum albumin as a standard.

Determination of 3'-AMP Forming Enzyme Activity 3'-AMP forming enzyme activity was determined as follows.

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The reaction mixture (55 µl) contained 100 mM acetate buffer (pH 5.8), 0—30 mM EDTA, 5—50 µg poly(A) and 5 µl of the crude enzyme solution. After incubation of the mixture at 37 °C for 60 min, the reaction was terminated by the addition of 55 µl of 15 mM ZnCl₂, in 0.5 M perchloric acid. The acid-soluble compounds were derivatized with chloroacetaldehyde for fluorescence detection.\(^{13}\)

RESULTS

In a preliminary experiment using 0.05 M acetate buffer (pH 4.5) containing methanol (96:4, v/v) as a mobile phase, separations of ATP and adenosine diphosphate (ADP) from AMP and Ado from cAMP were excellent, but that of 3'-dAMP from 3'-AMP was poor. As the final pH of the mobile phase changed from 4.2 to 5.0, the separation of 3'-dAMP from 3'-AMP was best at pH 4.5. A good separation of 3'-dAMP from 3'-AMP was obtained when the concentration of acetate buffer (pH 4.5) in the mobile phase containing 4% methanol was changed to 0.025 M. As the separation of these nucleotides increased with a decrease in the final % of methanol in the range of 10—1% in 0.025 M acetate buffer (pH 4.5), 3% methanol was selected for analysis of 3'-AMP in biological samples. As shown in Fig. 1A, the adenine compounds were well separated by isocratic elution on the reversed-phase HPLC column. ATP, ADP, AMP, 3'-dAMP, 3'-AMP, 2'-AMP, Ado and cAMP were eluted in this order and the analysis time was ca. 32 min. Detection limit at S/N=3 was ca. 15 pmol 3'-AMP. A good linearity for authentic 3'-AMP was observed in the range of 1.5—600 pmol. The established method was applied to determine adenine compounds in rat liver. The amounts of 3'-AMP, 2'-AMP, AMP and Ado were 23±7, 182±21, 1787±83 and 130±10 nmol/g wet weight (mean±S.E., \(n=5\)), respectively, but 3'-dAMP was not detectable under the conditions employed (Fig. 1B). The peak eluted just after 3'-AMP (Fig. 1B) was estimated to be adenine by comparison of the retention time of authentic adenine. The recovery of 3'-AMP added to liver homogenate was 103±7% (mean±S.D., \(n=6\)).

Our preliminary results showed that only two peaks, which corresponded to 3'-AMP and Ado, respectively, were detected when crude mitochondrial extract in 0.1 M phosphate buffer (pH 7.0) was incubated with poly(A) as a substrate in 0.1 M acetate buffer (pH 5.8). This finding prompted us to shorten the analysis time by changing the concentration of methanol. As shown in Fig. 2A, the analysis time for determination of 3'-AMP and Ado was ca. 7 min when 15% (v/v) methanol in the mobile phase containing 0.025 M acetate buffer (pH 4.5) was selected. After incubation of poly(A) with crude mitochondrial extract in 0.1 M phosphate buffer at 37 °C for 60 min, only two peaks corresponding to 3'-AMP and Ado, respectively, were detected (Fig. 2B). The enzymatic formation of 3'-AMP and Ado were determined to be 186±3 and 64±1 nmol/mg protein/60 min, respectively. To optimize the assay conditions for determining the activity of 3'-AMP forming enzyme, the biochemical properties were next examined. The optimum pH was determined to be around 5.8, as shown in Fig. 3. Addition of Zn²⁺ or p-chloromercuribenzoate (PCMB) to the reaction mixture completely abolished formation of both 3'-AMP and Ado (Table I). Formation of 3'-AMP and Ado was also signi-

Fig. 1. Chromatograms of Authentic Adenine Compounds (A) and Adenine Compounds from Rat Liver (B) on a Column of Chromatorex ODS
A) Forty micrometers of 5 µM adenine compounds in 0.1 M HEPES (pH 7.0) were injected. The relative fluorescence intensity (RFI) of the peaks was drawn.

Fig. 2. Chromatograms of Authentic Adenine Compounds (A) and Adenine Compounds in Reaction Mixture of Poly(A) and the Crude Enzyme Solution (B)
A) Twenty micrometers of 5 µM adenine compounds in 0.1 M HEPES (pH 7.0) were injected.

Fig. 3. Optimum pH of 3'-AMP Forming Enzyme in the Crude Enzyme Solution
Britton-Robinson wide-range buffer (pH 5.3—6.2) was used.
Table 1. Effects of Metal Ions, EDTA and PCMB on 3'-AMP Forming Enzyme Activity in Rat Crude Liver Mitochondrial Extract

<table>
<thead>
<tr>
<th>Concen. (mM)</th>
<th>3'-AMP (nmol/mg protein)</th>
<th>Ado (nmol/mg protein)</th>
<th>Total (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>185.9±2.8</td>
<td>63.5±0.5</td>
<td>249.4±2.3</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PCMB</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>61.5±2.9</td>
<td>27.2±0.8</td>
<td>88.7±1.7</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1170.0±6.8</td>
<td>38.1±1.0</td>
<td>1560.0±1.7</td>
</tr>
<tr>
<td>EDTA</td>
<td>315.3±12.7</td>
<td>118.5±6.4</td>
<td>433.8±19.8</td>
</tr>
<tr>
<td>Molybdate</td>
<td>247.8±7.8</td>
<td>2.9±0.2</td>
<td>250.7±7.8</td>
</tr>
</tbody>
</table>

Original reaction mixture contained no EDTA. The amount of poly(A) added to the mixture was 25 µg. All values are presented as means±S.E. of triplicates.

![Graph](image)

Fig. 4. Relationship between Incubation Time and Formation of 3'-AMP and Ado

- (O) Ado formed, (■) 3'-AMP formed, (○) total amount of 3'-AMP and Ado formed.

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DISCUSSION

We recently reported that 3'-AMP, a P-site inhibitor of adenylate cyclase, may be enzymatically released from RNAs in rat liver mitochondria. The nucleotide was mainly analyzed using a size exclusion-HPLC column of Asahipak GS-320H which could not separate 3'-AMP from 3'-dAMP, another P-site inhibitor of adenylate cyclase. Although several methods for determination of 3'-AMP have been developed by using HPLC with UV detection, the specificity for determination of 3'-AMP in biological samples is poor because of interference by a number of UV absorbing materials. The amount of rat liver 3'-AMP determined using fluorescent-HPLC, established in this study, was 23±7 nmol/g wet weight. No 3'-dAMP was detected in rat liver, suggesting that 3'-AMP may be the sole adenylic inhibitor of adenylate cyclase. Surprisingly, a large amount of 3'-AMP was found in rat liver; however, the physiological significance remains to be elucidated. The established fluorescent-HPLC method is suitable for determination of adenine compounds, including 3'-AMP, in biological samples.

Rat liver has been shown to possess both acid and alkaline RNase which have optimum pH values at 5.8 to 6.0 and at 7.8 to 8.0, respectively. Mitochondria are known to contain a site-specific endoribonuclease and a Mg²⁺ dependent poly(A) polymerase, which exonucleolytically releases AMP from poly(A). When rat mitochondrial extract was incubated with poly(A), only 3'-AMP and Ado were detected by reversed-phase HPLC column (Fig. 2B) and also by size-exclusion HPLC (data not shown). These results clearly demonstrated that 3'-AMP forming enzyme(s) existed in rat liver mitochondria. Generally, RNases are believed not to release Ado directly from RNAs. Rat liver mitochondria possess acid phosphatases and 5'-nucleotidases, which might catalyze conversion of 3'-AMP to Ado. The conversion of 3'-AMP to Ado was inhibited by molybdate (Table 1). This observation suggests that a molybdate-sensitive acid phosphatase coexisted in the rat mitochondrial extract. The 3'-AMP forming enzyme activity in the mitochondrial extract was not enhanced by PCMB treatment (Table 1), suggesting that the enzyme(s) was predominantly present in an active form, rather than an inactive or latent form as reported previously. The enzyme activity was independent of Mg²⁺ and was maximum at pH 5.8. From these results, 3'-AMP forming enzyme(s) in rat mitochondria could be classified as an acid exoribonuclease which has not been characterized so far.

The optimized assay method for determination of 3'-AMP forming enzyme may help us to study and discuss the physiological and pathophysiological role of 3'-AMP in living systems.

Acknowledgements The authors are grateful to Mr. N. Muto, Mr. H. Akagi and Miss R. Ogata of this University for their technical assistance.

REFERENCES