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Assay of ATPase and Na,K-ATPase activity using high-performance liquid chromatographic determination of ADP derived from ATP

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Abstract

An HPLC assay for determination of ATPase activity was developed and validated. After stopping the enzyme reaction of the enzyme source (rat renal cortical basolateral membranes) with ATP, products derived from ATP were analyzed by two methods; HPLC determination of ADP derived from ATP, and colorimetry of inorganic phosphorus (P_i) released from ATP. This HPLC procedure was precise and linear over the range of protein amount of the enzyme source studied, and the intra- and inter-assay variations were lower than 10%. The values that were obtained by the two methods revealed a significant correlation. Also, even when the samples contained P_i or were contaminated with P_i, this HPLC method allowed determination of ATPase activity. In addition, when ouabain was used as an inhibitor, the HPLC method was found to be applicable for Na,K-ATPase determination. This indicated that this HPLC assay would enable determination of ATPases other than Na,K-ATPase, when other inhibitors are employed instead of ouabain.

Keywords: ATPase; Na,K-ATPase; Enzymes; ADP

1. Introduction

To determine ATPase activity, a variety of methods including colorimetry of inorganic phosphorus (P_i) released from ATP, radiometry of the release of $^{32}P_i$ from [γ-^{32}P]ATP, regenerating systems for ATP, and a pH-metric procedure have been described [1]. The most commonly used of these methods is colorimetry of P_i. However, with this method care is required for the coloration reaction of P_i to avoid contamination of P_i in the reagent solutions and the experimental equipment. When samples contain P_i or are contaminated by P_i, the coloration reaction gives a relatively large error. In such cases, therefore, other methods should be employed. As an example, the release of $^{32}P_i$ can be measured using [γ-^{32}P]ATP [1]. Although this method is highly sensitive, it requires special equipment and is expensive.

We considered that since ATPase formed ADP from ATP through P_i release, measurement of ADP could be used as an alternative for the assay of ATPase activity. On the basis of this consideration,

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we used high-performance liquid chromatography (HPLC) to measure ADP derived from ATP by the enzymatic reaction of ATPase, and verified the efficacy of this procedure. In addition, we attempted to determine Na,K-ATPase using this HPLC method.

2. Experimental

2.1. Materials

AMP, ADP, and ATP were purchased from Boehringer Mannheim (Mannheim, Germany), ouabain was from Sigma (St. Louis, MO, USA), and tetrabutylammonium hydrogen sulfate was from Wako (Osaka, Japan). All other chemicals used were of analytical or HPLC grade.

For HPLC assay, a reversed-phase ODS column (Mightysil RP-18; 5 μm, 250×4.6 mm) and its guard column (Mightysil RP-18; 5 μm, 5×4.6 mm) were obtained from Kanto (Tokyo, Japan).

Male Sprague–Dawley rats weighing 200±10 g were obtained from Hokudo (Abuta, Hokkaido, Japan), housed in ordinary cages, and allowed free access to water and a standard pellet diet (CE-2; Clea Japan, Tokyo, Japan) prior to the study.

2.2. Preparation of basolateral membranes from the rat renal cortex

Rats were anesthetized with ether, and the kidneys were removed through a ventral incision. The renal cortices were homogenized according to the method of Inui et al. [2], and basolateral membranes were isolated by Percoll density gradient centrifugation. The basolateral membranes were routinely suspended in 250 m*M* sucrose–10 m*M* Hepes buffer (pH 7.4), frozen at −80°C until analysis, and used as an enzyme source for determination of ATPase and Na,K-ATPase activity.

2.3. Protein determination

Protein amount of the basolateral membranes was determined by the method of Bensadoun and Weinstein [3], in which the method of Lowry et al. [4] was modified so that proteins could be assayed in the presence of interfering substances.

2.4. Determination of ATPase and Na,K-ATPase activity

ATPase and Na,K-ATPase activities were determined by a modification of the method of Post and Sen [5]. For enzyme reaction, 0.1 ml of 1000 m*M* sodium chloride–100 m*M* potassium chloride, 0.2 ml of 250 m*M* Tris–acetate buffer (pH 7.2), 0.1 ml of 1 m*M* EDTA, 0.1 ml of 30 m*M* magnesium chloride, 0.1 ml of 10 m*M* ouabain (or water), x ml of the enzyme source, and $(0.3-x)$ ml of water, were put into glass tubes, and preincubated at 37°C for 5 min. Enzyme reaction was started by addition of 0.1 ml of 33.3 m*M* ATP, and the mixture was incubated at 37°C for 20 min. The enzyme reaction was stopped by addition of 2 ml of 1.5 *M* perchloric acid. After kept in ice-cold water for 20 min, the mixture was centrifuged (1700 *g*, 4°C, 10 min) to obtain the supernatant. Using the supernatant, the enzyme activity was determined by the two procedures mentioned below.

For colorimetric determination of P_i released from ATP, 0.5 ml of 4% sodium molybdate in 2.5 *M* sulfuric acid and 1 ml of 0.05% tin(II) chloride were added to 1 ml of the supernatant [6]. The mixture was kept at ambient temperature for 15 min, and absorbance was measured at 660 nm. P_i concentration was calculated from the regression line based on standard P_i solutions.

For HPLC determination of ADP derived from ATP, 0.1 ml of the supernatant was added to 0.9 ml of water. The mixture was further centrifuged (10 062 *g*, 4°C, 10 min), and the supernatant was used for the HPLC assay. Conditions used for HPLC were as follows: mobile phase, methanol–[10 m*M* tetrabutylammonium hydrogen sulfate in 20 m*M* ammonium dihydrogenphosphate–diammonium hydrogenphosphate (pH 6.5)] (25:75); flow-rate, 1.0 ml/min; column temperature, 40°C; wavelength for UV detection, 260 nm; injection volume, 20 μl; injection intervals, 20 min. The amount of ADP in each sample was calculated by measuring its peak area from the regression line based on the standard solution of ADP.

2.5. Calculation

ATPase activity was defined as the difference in activity of the sample and the sample blank; the sample blank was incubated without ATP, and ATP was added after the reaction was stopped by perchloric acid.

Na,K-ATPase activity was defined as the difference in activity in the presence and absence of ouabain.

One unit of ATPase and Na,K-ATPase activity was defined as 1 μmol of P_i released from ATP per min as well as 1 μmol of ADP derived from ATP per min.

The data are expressed as means±standard deviation (SD). Error rates (%) were calculated in SD× 100/mean. Correlation coefficient (*r*) was calculated by the standard method.

3. Results and discussion

3.1. HPLC of AMP, ADP and ATP

A representative chromatogram of AMP, ADP and ATP after incubation of ATP and enzyme source (25 μg protein/tube) is shown in Fig. 1. AMP, ADP and ATP were eluted at 4.2, 6.1 and 9.2 min, respectively, in the HPLC system employed.

3.2. Formation of ADP from ATP, and release of P_i from ATP

Formation of ADP from ATP, and release of P_i from ATP by the enzyme reaction were examined with addition of 0 and 5–100 μg of enzyme source per tube.

HPLC revealed that the amount of ADP increased linearly with the protein amount of the enzyme source (Fig. 2).

Colorimetric determination revealed that the amount of P_i released from ATP by ATPase increased with the protein amount of enzyme source (Fig. 3); linearity was found between 0 and 5–100 μg of the enzyme source per tube.

The correlation between the amount of P_i and the amount of ADP was estimated in this range over which linearity was observed in the two determinations (Fig. 4). There was a significant correlation between the values obtained by the two methods. Nevertheless, the values obtained by the HPLC determination were approximately 20% higher than

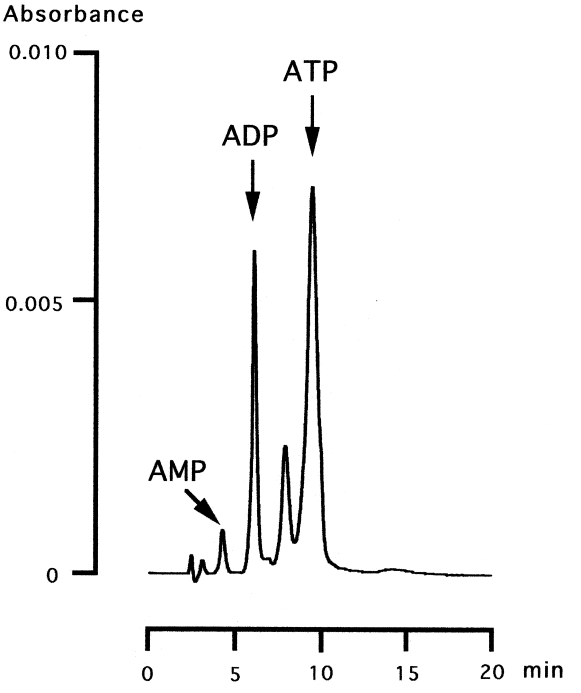

Fig. 1. Representative chromatograph of AMP, ADP and ATP after incubation of enzyme source with ATP. Enzyme source: renal cortical basolateral membranes (25 μg/tube). Conditions of enzymatic reaction and HPLC are described in the Section 2. Retention time (min): AMP, 4.2; ADP, 6.1; ATP, 9.2.

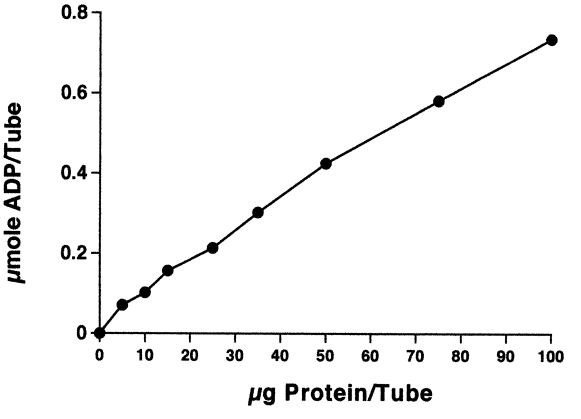

Fig. 2. HPLC determination of amount of ADP derived from ATP by ATPase. Enzyme source: renal cortical basolateral membranes.

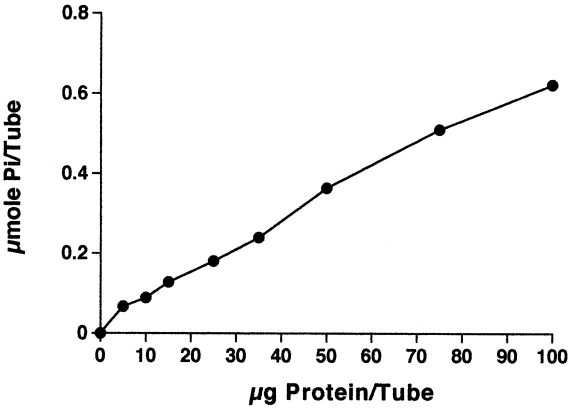

Fig. 3. Colorimetric determination of amount of P_i released from ATP by ATPase. Enzyme source: renal cortical basolateral membranes.

those determined by the colorimetric method. This difference was considered to be brought by the other enzymatic reactions in addition to the analytical errors. The enzyme source used was crude, and possibly contained the other enzymes and substrates. Accordingly, the following reactions were surmised to be more or less caused simultaneously when ATPase reaction was started: in terms of P_i, dephosphorylation and phosphorylation by the other enzymes; in terms of ADP, transformation of ADP to or from other adenosine phosphates by the other enzymes [1,5].

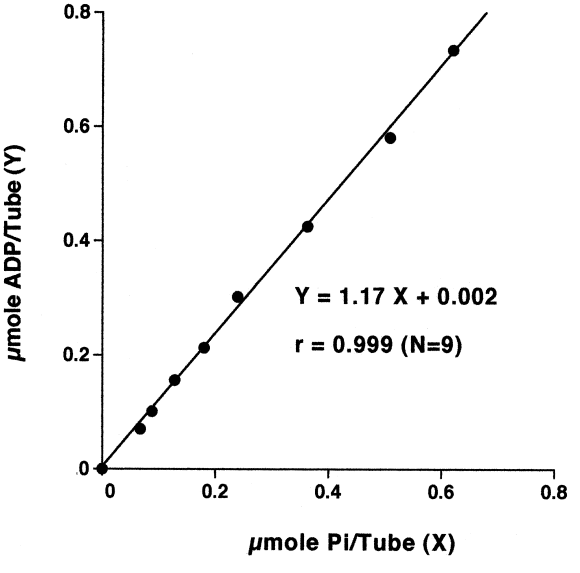

Fig. 4. Correlation between amount of P_i released from ATP and amount of ADP derived from ATP by ATPase. Enzyme source: renal cortical basolateral membrane. Amount of P_i (*x*-axis) derived from ATP and amount of ADP (*y*-axis) derived from ATP by ATPase shown in Figs. 2 and 3 are plotted. *n*, number of points; *r*, correlation coefficient.

3.3. Method validation

Using an identical enzyme source (25 μg/tube), precision in intra- and inter-day assays was estimated (Table 1). For the intra-day assay, the assay was repeated 6 times on the same day, while for the inter-day assay, the assay was performed once a day over the 6-day period. The intra- and inter-day precisions, expressed as percentages of the standard deviation of the observed mean activity, were less than 10% for both the colorimetric and the HPLC procedures.

3.4. Effects of P_i in aliquots

An identical enzyme source was suspended in two buffer solutions; the routine solution of 250 m*M* sucrose–10 m*M* Hepes buffer (pH 7.4), and 100 m*M* potassium phosphate buffer (pH 7.4). Protein amount of the enzyme source was adjusted to 0.5 mg/ml with the same buffer, and aliquots of 50 μl (25 μg/tube) were used for enzyme reaction. For the P_i determination method, absorbance of aliquots in the phosphate buffer was too high (more than 2.000) relative to the reagent blank, and therefore the activity could not be estimated (Table 2). In contrast, when the activity was determined by the HPLC

Table 1
Precision of ATPase activity determined by colorimetry of P_i released from ATP and by HPLC of ADP derived from ATP[a]

	ATPase activity (U/g protein)	
	Colorimetry of P_i	HPLC of ADP
Intra-day assay (n=6)	339.7±11.7	423.9±30.2
Error rate (%)	3.4	7.1
Inter-day assay (n=6)	348.8±25.0	439.7±38.9
Error rate (%)	7.2	8.8

[a] Enzyme source: renal cortical basolateral membranes (25 μg/tube). Each assay was performed using an identical enzyme source. Values: means±standard deviation (SD). Error rate (%): SD×100/mean.

Table 2
Effects of P_i in samples on determination of ATPase activity using colorimetry of P_i released from ATP and HPLC of ADP derived from ATP[a]

Medium containing enzyme source	ATPase activity (U/g protein)	
	Colorimetry of P_i	HPLC of ADP
250 m*M* Sucrose–10 m*M* Hepes buffer (pH 7.4)	370.0	466.2
100 m*M* Potassium phosphate buffer (pH 7.4)	Not determined	491.6

[a] Enzyme source: renal cortical basolateral membranes. An identical enzyme source was prepared at a protein concentration of 0.5 mg/ml in the above two buffer solutions. Fifty-μl aliquots were added to tubes: protein amount, 25 μg/tube. Values: means of two determination. 'Not determined': excessively high absorbance (>2.000) relative to reagent blank.

method, the values of the two aliquots were nearly equivalent (Table 2). This indicated that the HPLC method enabled determination of ATPase activity when the samples contained P_i or were contaminated with P_i.

3.5. Applicability of the HPLC method for the other ATPases

Renal cortical basolateral membranes were obtained from six rats, and Na,K-ATPase activity was determined by the two methods described above (Table 3). Similarly to the ATPase, the activity of the Na,K-ATPase determined by the HPLC method was approximately 20% higher than the value determined by the colorimetric method, and the HPLC method was found to be applicable for determination of Na,K-ATPase activity. Thus, we considered that when the other inhibitors, e.g., *N*-ethylmaleimide [7], bafilomycin [8], etc., are used instead of ouabain, our HPLC method would allow determination of other ATPases.

Table 3
Na,K-ATPase activity determined by colorimetry of P_i released from ATP and by HPLC of ADP derived from ATP[a]

Na,K-ATPase activity (U/g protein)	
Colorimetry of P_i	HPLC of ADP
103.5±14.8	127.8±21.1

[a] Enzyme source: renal cortical basolateral membranes (25 μg/tube). Each assay was performed using different enzyme sources obtained from six rats. Values: means±standard deviation (SD).

4. Conclusions

A procedure based on HPLC for quantifying ADP was developed and validated for determination of ATPase activity in rat renal cortical basolateral membranes. This procedure was precise and gave linear results over the range of protein amount of the enzyme source examined. Also, the procedure was found to be hardly affected even when the sample contained P_i or was contaminated with P_i. In addition, when ouabain was used as an inhibitor, the method was found to be applicable for determination of Na,K-ATPase activity. Thus, this method would allow determination of other ATPases, providing that other inhibitors were employed instead of ouabain.

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