Bioluminometric Method for Real-Time Detection of ATPase Activity

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ABSTRACT

We have developed a real-time, simple, and sensitive method for the detection of ATP hydrolysis activity (ATPase) of apyrase (EC 3.6.1.5). The assay is based on the continuous monitoring of the ATP hydrolysis reaction using the firefly luciferase system. The method is sensitive and yields linear responses between 0.7 and 70 mU for the *Solanum tuberosum* apyrase. The detection limit was found to be 0.7 mU apyrase. We used the method to study the inhibitory effects of various compounds on the ATPase activity of potato apyrase, measured with 500 nM ATP. The concentrations of azide, AMP, P_i , fluoride, and ADP, which inhibit the ATPase activity by 50% (IC_{50}), were found to be approximately 100, 0.25, 0.125, 0.04, and 0.035 mM, respectively. Under our assay conditions, vanadate inhibited about 98% of the ATPase activity of the potato apyrase at a concentration of 250 μ M. The possibility of using the new method for other applications is discussed.

INTRODUCTION

Ectoapyrases and ecto-ATPases are members of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTDPase) family of enzymes (25). E-NTDPases are strictly dependent on divalent cations for activity and are generally insensitive to the specific inhibitors of P-type (ouabain and vanadate), F-type (azide, oligomycin, and dicyclohexylcarbodiimide) and V-type (N-ethylmaleimide and NBD-Cl) ATPases (20,32). Ectoapyrases are distinguished from ecto-ATPases by their ability to hydrolyze both nucleoside tri- and diphosphates and their inhibition by 10–20 mM azide (25). E-NTDPases have been isolated and studied from animal sources and organisms such as rat secretory tissue (30), rat hepatocytes (21,22), rabbit skeletal muscle transverse tubules (29), bovine lung (24), *Toxoplasma gondii* (4), and yeast (26). They have also been isolated from plant tissues such as pea stem (27), chick-pea root (31), and potato tuber (15,16). Apyrases isolated from *Solanum tuberosum* are among the most extensively studied apyrases, and several isoforms have been isolated (17). It has been suggested that E-NTDPases participate in neurotransmission (5), blood platelet aggregation and blood pressure regulation (23), protein glycolysation (1), and the regulation of membrane integrity (8). The molecular properties of this family of enzymes have been recently described (33).

Both colorimetric- and radioisotope-based methods have been developed and used for the detection of ATPase activity, based on the measurement of

the product released from the apyrase reaction, either orthophosphate or ADP (2,6,9,19,28).

Interestingly, the coupled reaction that links ADP production to NADH oxidation (3) has not been used with the E-NTDPases, although it is capable of measuring the activity of 50 mU enzymatic activity.

Most of these methods are labor intensive, require time-consuming procedures, and do not detect the ATPase activity in real time. Here, we describe a simple and sensitive technique for the detection of ATPase activity in real time using the luciferase/luciferin system.

MATERIALS AND METHODS

Real-Time ATPase Method

The standard assay volume (0.2 mL) contained the following components: 0.1 M Tris-acetate, pH 7.75, 2 mM EDTA, 10 mM magnesium acetate, 0.1% BSA, 1 mM DTT, 0.4 mg/mL polyvinylpyrrolidone ($M_r = 360\,000$), 100 μ g/mL D-luciferin (the last four products were from Sigma, St. Louis, MO, USA), and purified luciferase (Promega, Madison, WI, USA) in an amount giving a response of 10 mV for 0.1 μ M ATP (about 3 ng luciferase). After the addition of potato apyrase (Sigma), the reaction was initiated by the addition of 100 pmol ATP. The luminescence was measured using a LKB 1250 tube luminometer connected to a poten-

Table 1. The Effect of Various Inhibitors on the Potato Apyrase-Catalyzed ATPase Activity

NaN ₃		P _i		AMP		ADP		NaF	
Conc. (M)	R.A. (%)	Conc. (mM)	R.A. (%)	Conc. (mM)	R.A. (%)	Conc. (μM)	R.A. (%)	Conc. (mM)	R.A. (%)
0	100	0	100	0	100	0	100	0	100
0.1	50	0.005	93	0.05	80	2.5	80	0.01	90
0.16	40	0.025	75	0.125	64	5	72	0.045	60
0.4	20	0.125	50	0.25	50	25	40	0.115	37.5
1.2	9	0.5	25	0.5	35	75	20		
		2.5	8	2.5	8.3				
				5	1				

There were 7 mU apyrase and 100 pmol of ATP in each reaction. The table shows the concentration of each inhibitor (Conc.) and the percentage of the apyrase-catalyzed ATP hydrolysis activity (R.A.). Each point was obtained from individual assays and represents an average value from several experiments. The experimental conditions were as described in Materials and Methods.

tiometric recorder, and the luminometer was calibrated to give a response of 10 mV for the luminometer internal standard.

To study the effects of vanadate (0–0.5 mM), azide (0–1.25 M), fluoride (F[−]) (0–0.11 mM), AMP (0–5 mM), and P_i (0–2.5 mM) (all inhibitors were from Sigma Chemical), several assays were performed. The inhibitor and 7 mU apyrase, one unit liberates 1 μmol P_i/min at 30°C, were preincubated in

the standard assay mixture before the reaction was started by the addition of 100 pmol ATP and the luminescence was recorded. To study the effect of ADP (0–0.1 mM), several assays were performed. The standard assay used was as previously described, with the exception that the reaction was started by the addition of both ATP and ADP (contaminated with ATP) so that the total amount of ATP in the reaction was 100 pmol. The effect of the inhibitor on the

luciferase reaction was established by measuring the light yield of ATP in the presence of the inhibitor (Figure 1A); in all cases, the inhibitors did not affect the reaction of luciferase with ATP.

RESULTS

Real-Time Detection of ATPase Activity

Potato apyrase (or any enzymatic activity that hydrolyzes ATP) is preincubated with the reaction mixture before the reaction is started by the addition of ATP. The assay reactions are shown below.

The concentration of ATP was determined by the luciferase reaction (Figure 1A) [Eq. 2]. Under our assay conditions, the amount of light produced is proportional to the concentration of ATP in the range of 10 pM–1 μM. At higher concentrations of ATP, the reaction is complicated by the inhibition of the luciferase by oxyluciferin (7). The amount of ATP consumed in the luciferase reaction is negligible because the light emission is constant

Figure 1. Typical traces from real-time monitoring of apyrase activity. Apyrase was either omitted (A) or preincubated (B) in the assay mixture before the reactions were started by the addition of 100 pmol ATP at a final concentration of 0.5 μM. The ATP degradation was detected by the luciferase system. The experimental conditions were as described in Materials and Methods.

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over a period of more than 10 min. The activity of potato apyrase was measured as the decrease in the ATP concentration detected by luminescence as a function of time (Figure 1B). The loss of ATP appears to follow an exponential curve. When these data were fitted with the software program "Igor" (WaveMetrics, Lake Oswego, OR, USA), the decay curve appeared to be an exponential curve with a value of $k_{app} = 0.0991 \text{ s}^{-1}$ (Figure 2).

Because the K_m value for ATP of potato apyrase is $200 \text{ } \mu\text{M}$ (25) and the concentration of ATP in the experiment was 500 nM , the kinetic equation for the enzymatic reaction:

is the first order part of the Michaelis-Menten equation. The initial rate, v , is given by

$$v = \frac{-d(S)}{dt} = (S)(Etot) \frac{k_{cat}}{K_m} \quad [\text{Eq. 5}]$$

where (S) is the concentration of ATP, $(Etot)$ is the total apyrase concentration, and K_m and k_{cat} have the usual significance. The decay of the substrate concentration (S) is given by

$$(S) = (So)e^{-\frac{(Etot)k_{cat}}{K_m}t} = (So)e^{-k_{app}t} \quad [\text{Eq. 6}]$$

where (So) is the initial concentration of ATP and

$$k_{app} = (Etot) \frac{k_{cat}}{K_m} \quad [\text{Eq. 7}]$$

Because $t_{0.5} = \frac{0.693}{k_{app}}$ for a first-order reaction, we report the values of $t_{0.5}$ for the reactions.

Effect of ATP Concentration

We used this method to study the effect of ATP concentration on the ATPase reaction catalyzed by apyrase. Figure 3 shows v (from equation 5) for ATP degradation catalyzed by apyrase as a function of the ATP concentration; v is directly proportional to ATP, between 0 and 500 nM .

On the basis of this experiment, ATP at a final concentration of 500 nM was chosen as an optimal concentration for

the sensitive detection of ATPase activity. The ATP decay rate was proportional to the amount of apyrase, as predicted by Eq. 7 (Figure 4). Using this method, we were able to detect ATPase activity from potato apyrase between 0.7 and 70 mU . Because the rate of consumption of ATP by luciferase is very slow (Figure 1A), it is possible to measure the activity of 0.07 mU apyrase ($t_{0.5} = \text{approximately } 3 \text{ min}$). It is worth noting that the unit is defined for a reaction performed at 30°C and $\text{pH } 6.5$ for the potato apyrase, whereas our experiments were performed at room temperature, about 22°C , and $\text{pH } 7.75$.

Inhibition of the ATPase Activity by Vanadate and Other Inhibitors

This method can be used to study the effects of different substrates on the ATPase activity. For example, vanadate is not known to inhibit ectoapyrase activity; however, we have detected an inhibitory effect of vanadate on the ATPase activity of apyrase from *S. tuberosum* (Figure 5). Nearly 50% inhibition was obtained at a concentration of about $20 \text{ } \mu\text{M}$. At 0.5 mM vanadate, about 98% inhibition of the apyrase ATPase activity was observed (Figure 6). The assay has also been used to

Figure 2. The apyrase ATPase activity as a function of time. The reactions were started by the addition of 100 pmol ATP to an assay mixture containing $7 \text{ } \mu\text{U}$ *S. tuberosum* apyrase. The ATP degradation was detected by the luciferase system. Each point was obtained from individual assays and represents an average value from several experiments. The data were fit with the equation shown in the caption with the Igor program: fit_wave1 is the value of luminescence at time t , W_coef[1] is the initial value of luminescence, W_coef[2] represents k_{app} , and W_coef[0] is the residual fluorescence at the end of the reaction. The values of W_coef are given in the second line of the caption, and the statistical values of the fit are given in lines 3 and 4. The experimental conditions were as described in Materials and Methods.

study the effect of other inhibitors on the ATPase activity of the potato apyrase (Table 1). The IC_{50} for azide, AMP, P_i , F^- , and ADP were found to be approximately 100, 0.25, 0.125, 0.04, and 0.035 mM, respectively. In contrast, with the conventional assay, 2 mM F^- and 0.2 mM vanadate had no effect on the ATPase activity of chicken oviduct ecto-ATPase, while 10 mM azide inhibited 70%–74% of the activity (18).

DISCUSSION

Generally, bioluminescence-based techniques have several advantages over commonly used methods, such as speed, simplicity, and sensitivity. Therefore, new bioluminescence-based methods continue to be developed (12–14). Here, we present a bioluminometric approach for the real-time detection and quantification of ATPase activity.

Figure 3. The initial rate of apyrase-catalyzed ATP degradation as a function of the ATP concentration. The reactions were started by the addition of the ATP (at the indicated final concentration) to an assay mixture containing 7 μ U *S. tuberosum* apyrase. The ATP degradation was detected by the luciferase system. Each point was obtained from individual assays and represents an average value from several experiments.

Figure 4. Apparent rate constant (k_{app}) of apyrase-catalyzed ATP hydrolysis as a function of the apyrase amount. The reactions were started by the addition of 100 pmol ATP to an assay mixture containing the indicated amount of apyrase. ATP hydrolysis was detected by the luciferase system. Each point was obtained from individual assays and represents an average value from several experiments. In the inset, the k_{app} obtained at low apyrase concentrations are shown. The experimental conditions were as described in Materials and Methods.

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Figure 5. The apyrase ATPase activity as a function of vanadate concentration. Apyrase and vanadate were preincubated in the reaction mixture. The reaction was started by the addition of ATP at a final concentration of 0.5 μ M. The ATP hydrolysis was detected by the luciferase system. Each point was obtained from individual assays and represents an average value from several experiments. One hundred percent represents the maximum value of apyrase ATPase activity in the absence of vanadate. The experimental conditions were as described in Materials and Methods.

Figure 6. Typical trace obtained from real-time monitoring of the inhibitory effect of vanadate on the apyrase-catalyzed ATPase activity. ATP (100 pmol) was added in the presence of 7 μ U apyrase. When almost all of the added ATP had been hydrolyzed by apyrase, vanadate at a final concentration of 300 μ M was added. ATP (100 pmol) was then added to the reaction. ATP hydrolysis was detected by the luciferase system. The experimental conditions were as described in Materials and Methods.

Similar to most of the luminescent assays, this is a simple, one-step method involving the addition of apyrase to the reagents. There is no need for incubation steps or the addition of stopping or developing solutions. The reaction can be completed within a minute, which allows fast analysis of many samples.

The real-time ATPase approach has a broad dynamic range, and the detection limit is approximately 0.7 mU potato apyrase. The sensitivity of the assay can be increased by performing the reaction in a smaller volume. At present, the assay is performed at room temperature because of the temperature sensitivity of the luciferase. However, the method's sensitivity can be increased by using a more thermostable luciferase (10,11) and by performing the assay at a higher temperature, which allows the optimal temperature for the apyrase.

The method has many possible applications, such as the determination of the specific activity for different enzyme batches and for the study of the effects of different inhibitors on the ATPase activity (Table 1). Furthermore, the developed method was used for the detection and quantification of both recombinant 5'-nucleotidase and CD39 ATPase activity during both the production and purification of the enzymes (data not shown). The method has also been used during the purification of potato apyrase (data not shown).

In this study, the real-time monitoring of the ATPase activity was performed with an LKB single-tube luminometer. However, by using a microplate-format luminometer, the capacity of the technique can be further increased.

In conclusion, a simple, real-time, nonradioactive method for the continuous measurement of ATPase activity has been developed. The method offers the possibility of studying the ATPase activity from different sources (e.g., ectoapyrases) in real time.

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