Method for Real-Time Detection of Inorganic Pyrophosphatase Activity

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A sensitive and simple method for real-time detection of inorganic pyrophosphatase (PPase) (EC 3.6.1.1) activity has been developed. The method is based on PPase-induced activation of the firefly luciferase activity in the presence of inorganic pyrophosphate (PPi). PPi inhibits the luciferase activity, but in the presence of PPase the luciferase activity is restored and the luminescence output increases. The assay yields linear responses between 8 and 500 mU. The detection limit was found to be 8 mU PPase. The method was used to detect the hydrolytic activity of PPases from Saccharomyces cerevisiae, Escherichia coli, and Bacillus stearothermophilus. As substrate for the luciferase, adenosine 5′-phosphosulfate can replace ATP, which is an advantage for detection of PPase activity in crude extracts containing ATP-hydrolyzing activities. The method can be used for kinetic and inhibition studies as well as for detection of PPase activity during different purification procedures.

Key Words: inorganic pyrophosphatase; inorganic pyrophosphate; inhibition; bioluminescence; luciferase; ATP; APS; luciferin.

Two major classes of inorganic pyrophosphatases (PPases; EC 3.6.1.1), soluble and membrane-bound, have been described (1–3). Soluble PPases are ubiquitous enzymes that catalyze specifically the hydrolysis of inorganic pyrophosphate (PPi) to orthophosphate (Pi). They are essential for life, providing a thermodynamic pull for many different biosynthetic reactions. Membrane-bound PPases catalyze the synthesis (photosynthetic bacteria type) or hydrolysis (vacuolar type) of PPi. The synthesis or hydrolysis of PPi catalyzed by the membrane-bound type is coupled to proton translocation over a membrane. Membrane-bound PPases are larger and do not show any sequence similarity to soluble PPases (4). The soluble class of PPases are the best studied and the crystal structure of the enzymes from Saccharomyces cerevisiae (5), Escherichia coli (6, 7), and Thermus thermophilus (8) have been determined.

Several assays have been developed for the detection of PPase activity. The enzymatic activity can be followed in either the synthesis or hydrolysis direction. The most convenient assay in the synthesis direction is the continuous bioluminometric method (9), whereas colorimetric (10) and enzymatic (11, 12) techniques are often employed for detection of the hydrolytic activity. Only two methods for continuous detection of hydrolytic activity have been described (13, 14). One method is based on the continuous detection of the change in the spectrum of methyl green in the presence of phosphate (13) and the other is based on the measurement, by a pH meter, of small pH changes caused by decrease of hydrogen-ion concentration during the PPi hydrolysis reaction (14). There is a clear need for simpler and more robust methods for real-time detection of PPi hydrolysis activity.

In this paper we describe a new method for real-time detection of PPase activity. The method is based on PPase-induced activation of the firefly luciferase activity in the presence of PPi.

MATERIALS AND METHODS

The PPase Assay

The standard assay volume was 0.2 ml and contained the following components: 0.1 M Tris–acetate (pH 7.75), 0.5 mM EDTA, 10 mM magnesium acetate, 2 mM dithiothreitol, 0.4 mg/ml polyvinylpyrrolidone.
(360,000), 25 μM inorganic pyrophosphate (Na₄P₂O₇) (the last three products from Sigma Chemical Co., St. Louis, MO), 0.1% bovine serum albumin, 100 μg/ml D-luciferin, 4 μg/ml L-luciferin, and 30 ng recombinant luciferase (the last four products from BioThema AB, Dalarö, Sweden). After the addition of ATP or adenine 5'-phosphosulfate (APS) to final concentrations of 0.2 μM and 0.5 mM, respectively, the reaction was started by addition of PPase (EC 3.6.1.1) from S. cerevisiae, E. coli, or Bacillus stearothermophilus (all enzymes from Sigma Chemical Co.). One unit of S. cerevisiae PPase will liberate 1.0 μmol of inorganic orthophosphate per minute at pH 7.2 and 25°C (according to Sigma Chemical Co.). The luminescence was measured using an LKB 1250 tube luminometer connected to a potentiometric recorder, and the luminometer was calibrated to give a response of 8 mV for the luminometer internal standard.

The sensitivity of the assay was determined in the presence of PPI and ATP at final concentrations of 25 and 0.2 μM, respectively. When the inhibition of NaF was studied, the inhibitor was preincubated in the standard assay before the reaction was started by the addition of PPase.

Luciferase Inhibition Study

When the effect of PPI on the luciferase activity was studied, several assays were performed. The standard assay used was as described above with the exception that the concentrations of ATP, APS, and PPI were varied. After the addition of ATP or APS, PPI was added and the light inhibition was recorded.

RESULTS

Principle of the PPase Assay

The PPase assay is based on the earlier observation (not published) that PPI reversibly inhibits the firefly luciferase reaction. In the assay, ATP (or any substance that functions as a substrate for the luciferase) is preincubated with PPI before the reaction is started by the addition of PPase (or any enzymatic activity that hydrolyzes PPI). The ATP-induced luminescence is partially inhibited in the presence of PPI. When the PPase is added, the PPI is continuously hydrolyzed, which can be monitored as an increase in luminescence. In an optimized assay the initial rate increase of the luciferase reaction after the PPase addition is proportional to the amount of PPase added.

The rate of the luciferase reaction [1] is proportional to the ATP concentration over the range 10 pM to 1 μM and the light emission is essentially time-independent.

\[
\text{ATP + D-luciferin + O}_2 \rightarrow \text{AMP} \\
+ \text{PPI} + \text{oxyluciferin} + \text{CO}_2 + h\nu \quad [1]
\]

Stable light is achieved by using low amounts of luciferase and by the presence of the competitive inhibitor L-luciferin at a 1 to 25 molar ratio with the D-isomer (15).

Effect of PPI on the Firefly Luciferase Reaction

The effect of PPI on the ATP- and APS-induced luminescence at fixed ATP and APS concentrations is shown in Fig. 1. At a final concentration of 45 μM PPI, the luciferase reaction was inhibited by approximately 50%. This result is in accordance with the earlier observation by Ford et al. (16). It is worth noting that Ford et al. found that PPI inhibited the luciferase activity at low ATP concentrations, whereas at high ATP concentrations PPI stimulated the activity. The inhibition was independent of the ATP and APS concentration in the range between 0.05 and 0.4 μM and 0.06 and 2 mM, respectively (not shown). The slope of the inhibition curve was largest for concentrations of PPI between 25 μM.

Real-Time Detection of PPase Activity

From the PPI inhibition experiments presented above we observed that the highest sensitivity of the assay was obtained at PPI concentrations below 25 μM. As the Kₘ values (for PPI) for most PPases are in the range between 0.5 and 5 μM (17–21), 25 μM was chosen as an optimal concentration for sensitive detection of PPase activity. Figure 2 shows typical traces obtained after addition of PPase to the assay. The change in light emission, as detected by a luminometer, was proportional to the amount of PPase added. Both ATP (Fig. 2A) and APS (Fig. 2B) could be used as substrates for the luciferase reaction. Under our assay conditions, we were able to detect 8 mU PPase from S. cerevisiae.
A linear relation between the PPI hydrolysis rate and the amount of PPase between 8 and 500 mU was observed (Fig. 3). The rate of the reaction was calculated from the relation between the luminescence activity and the PPI concentration as described in the legend to Fig. 1. It is worth noting that the unit is defined for a reaction performed at 25°C and pH 7.2 for the yeast enzyme, whereas our experiments were performed at room temperature (about 20°C) and pH 7.75.

The PPase method can in principle be used for detection of all types of inorganic pyrophosphatases, although it might not be possible to use optimal reaction conditions for all enzymes. For instance, we were able to detect the activity of PPases from both *E. coli* and *B. stearothermophilus* (data not shown).

**Inhibition by NaF**

NaF is well known as an inhibitor of PPase activity. Under our assay conditions, 10 mM NaF inhibited the PPase activity by 80% (not shown). This value was corrected for the NaF-induced inhibition (about 15%) of the luciferase activity.

**DISCUSSION**

Here we present a bioluminometric approach for detection of PPase activity in real time. The assay is a simple one-step assay involving simply the addition of PPase to an assay mixture containing both ATP and PPI at final concentrations of 0.2 and 25 μM, respectively. In the inset, the rates obtained at high PPase concentrations are shown. The luminescence output was measured with a tube luminometer. Each point was obtained from individual assays and represents an average value from at least five experiments (the variation was about 5%). The experimental conditions were as described under Materials and Methods.

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**FIG. 2.** Typical traces obtained from real-time monitoring of PPase activity. ATP (A), APS (B), and PPI (A and B) at final concentrations of 0.2, 500, and 25 μM, respectively, were preincubated in the assay mixture before the reaction was started by addition of 8 mU *S. cerevisiae* PPase. The luminescence was measured with a tube luminometer. The ATP and PPI addition was done with the luminometer in the opened position, whereas the PPase addition was done in the closed position. The experimental conditions were as described under Materials and Methods.

**FIG. 3.** The initial PPI-hydrolyzing rate as a function of PPase (from *S. cerevisiae*) concentration. The reactions were started by the addition of PPase to an assay mixture containing both ATP and PPI at final concentrations of 0.2 and 25 μM, respectively. In the inset, the rates obtained at high PPase concentrations are shown. The luminescence output was measured with a tube luminometer. Each point was obtained from individual assays and represents an average value from at least five experiments (the variation was about 5%). The experimental conditions were as described under Materials and Methods.
pyrosequencing (22), were analyzed for the possibility of devastating PPase contamination (data not shown). The PPase assay is very flexible; with minor modifications (e.g., replacing of ATP with APS), the assay can be used for detection of PPase activity in the presence of ATP-hydrolyzing activities, although the presence of ATP sulfurylase activity will disturb the assay if APS is used. Further applications for the assay can be anticipated, such as for screening of new inhibitors.

In conclusion, a simple, real-time, nonradioactive assay for continuous detection of PPase activity has been developed. The assay has advantages that open up new possibilities to study the PPase in real time. The simplicity of the method makes it particularly suitable for future automation.

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REFERENCES