Characterization of Nucleoside Triphosphatase Activity in Isolated Pea Nuclei and Its Photoreversible Regulation by Light

YUH-RU CHEN and STANLEY J. ROUX*
Department of Botany, University of Texas at Austin, Austin, Texas 78713

Received for publication December 6, 1985 and in revised form March 1, 1986

ABSTRACT

A nucleoside triphosphatase (NTPase) present in highly purified preparations of pea nuclei was partially characterized. The activity of this enzyme was stimulated by divalent cations (Mg2+ = Mn2+ > Ca2+), but was not affected by the monovalent cations, Na+ and K+. The Mg2+-dependent activity was further stimulated by concentrations of Ca2+ in the low micromolar range. It could catalyze the hydrolysis of ATP, GTP, UTP, and CTP, all with a pH optimum of 7.5. The nuclear NTPase activity was not inhibited by vanadate, oligomycin, or nitrate, but was inhibited by relatively low concentrations of quercetin and the calmodulin inhibitor, compound 48/80. The NTPase was stimulated more than 50% by red light, and this effect was reversed by subsequent irradiation with far-red light. The photoreversibility of the stimulation indicated that the photoreceptor for this response was phytochrome, an important regulator of photomorphogenesis and gene expression in plants.

Several ATPases in plant organelles and membranes have been identified. These enzymes regulate ion transport and other functions in mitochondria (13), chloroplasts (22), Golgi (5), vacuoles (1), plasma membranes (16), and nuclei (20).

The role of NTPase activity in regulating the functions of plant and animal nuclei is not well understood, but past studies have tended to emphasize its influence on RNA synthesis and transport. For example, Grossman et al. (12) report that a NTPase in the nuclei of parsley cells helps to regulate the precursor pools for DNA-dependent RNA synthesis, and Agutter et al. (2) correlate the activity of a nuclear-envelope NTPase with the efflux of RNA from isolated nuclei of SV40-3T3 cells.

Recent reports have provided information on the location and regulation of plant nuclear ATPase activities. Jian et al. (17) used histochemical methods to detect ATPase activity in the nucleolus and chromatin of tomato roots. Wagle (31) found that R stimulates the activity of a Mg2+-dependent ATPase in pea nuclei and that FR reverses this effect. Metabolic responses that are photo-reversible by R and FR are considered to be under the control of the photochromic pigment, phytochrome, an important regulator of photomorphogenesis and gene expression in plants (15). In another study on the regulation of nuclear ATPases, Matsumoto et al. (20) demonstrated that calcium and calmodulin modulate an ATPase activity in the chromatin fraction of pea nuclei.

The reports of Wagle (31) and Matsumoto et al. (20), taken together, suggest that phytochrome and calmodulin may co-regulate a nuclear ATPase activity in pea nuclei. Resolving this question would provide new insights on the role of Ca2+ in mediating phytochrome responses (27) and refine ideas on the biochemical steps by which light is able to regulate gene activity in higher plants (30). To further investigate this matter, we have partially determined the biochemical characteristics of a pea nuclear NTPase and tested the responsiveness of this activity to light, Ca2+, and calmodulin inhibitors.

MATERIALS AND METHODS

The seeds of peas (Alaska) were germinated on fine vermiculite and grown in the dark at 26°C for 7 d. Only the plumules of the seedlings were used as the source tissue. The method of isolation of nuclei was essentially that of Hagen and Guilfoyle (14), as modified by Datta et al. (7). The plumules were exposed to green light for about 20 min during the harvesting. This light contained a low ‘‘contaminating’’ fluence (8 × 10⁻¹¹ mol/cm²) of actinic light in the 600 to 700 nm range. All extraction procedures were carried out at 4°C. The final pellet of purified nuclei was resuspended in Buffer R (60 mM Hepes [pH 7.5], 20% glycerol, 6.0 mM MgSO4, 10 mM 2-mercaptoethanol).

Percoll, ATP (Tris salt), GTP (disodium salt), CTP (sodium salt), UTP (disodium salt), ADP (sodium salt), oligomycin, quercetin, and compound 48/80 were obtained from Sigma Chemical Co. All other chemicals were of reagent grade.

Assay of NTPase Activity. After Percoll was removed, purified nuclei were resuspended, at a concentration of 1.3 to 1.5 mg protein/ml, in Buffer R plus 0.2 mM ammonium molybdate and 1.14 mM ascorbic acid to inhibit phosphatases. They were incubated in this buffer for 20 min at 4°C, then 0.1 ml of nuclei were added into 2 ml of the assay mixture (60 mM Hepes [pH 7.5], 20% glycerol, 6 mM MgSO4). The reaction was begun with the addition of Tris-ATP or other nucleotides, as indicated, to a final concentration of 3 mM. Reactions were continued for 15 min at 28°C and stopped by adding an equal volume of ice-cold 10% (v/v) TCA. The reaction mixture was centrifuged at 5000 rpm (rotor 221, International Clinical Centrifuge) for 10 min, and the released phosphate in the supernatant was determined by the method of Sanui (29). In this method, the released phosphate was reacted with molybdate ions in acid solution and the resulting phosphomolybdate complex was extracted into n-butyl acetate. The phosphomolybdate in the n-butyl acetate phase was separated from the assay mixture by centrifugation, then the absorbance of phosphomolybdate was measured at 310 nm. The amount of released phosphate was calculated from a standard curve. Each experiment was conducted with duplicate samples and with a buffer control. The Pi values were calculated from the average values of the released Pi in the duplicates minus the Pi amount of the buffer control.

1 Supported by grants from the National Science Foundation (PCM 8402526) and the National Aeronautics and Space Administration (NSG 7480) to S. J. R.

2 Abbreviations: NTPase, nucleoside triphosphatase (utilizes ATP and other nucleoside triphosphates as substrates); R, red light; FR, far-red light.
**Protein Assays.** Protein was assayed by the method of Lowry *et al.* (19) after solubilization of the nuclei with 5% (w/v) SDS. As a standard, BSA in 5% SDS was used.

**Inhibitor Studies.** For the inhibitor studies with vanadate, nitrate, and oligomycin, the inhibitor was added at the same time the nuclei were added to the assay buffer. The concentrations tested were equal to those shown to be effective in blocking other plant ATPases in previous reports (see references in "Results" section). When quercetin and EGT A were used, they were included during the 20 min preincubation in the resuspension buffer before the assay was begun. For studies with the calmodulin inhibitor, compound 48/80, the nuclei were preincubated with the drug for 30 min before starting the ATPase assay. When the inhibitor was added in an ethanolic solution, ethanol was added to the controls.

**Replication of Results.** Each experiment was performed in duplicate, and all data given here are the average of duplicate readings. The error bars shown on each figure represent the range of difference (almost always less than 10%) for duplicates in each experiment. Each experiment was repeated at least once, and most were repeated at least three times, with similar results.

**Irradiations.** The nuclei were incubated with 2 min actinic R and/or 2 min FR while they were in the assay buffer, just before substrate addition. The actinic R source produced an irradiance of approximately 15 µW cm⁻²; the actinic FR source produced an irradiance of about 24 µW cm⁻². The interference filters had 15 nm band widths and a λ_max of 665 nm (R filter) or 722 nm (FR filter). All irradiance values were obtained with an International Light Spectroradiometer. The ATP substrate was added immediately after the nuclei were irradiated.

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**RESULTS**

**Nuclei Preparation.** After centrifugation in the Percoll gradient, nuclei were concentrated at the interface of the 25 and 50% layers; debris remained at the top of 25% Percoll layer; starch grains were precipitated in the pellet at the bottom of the gradient. Examination by EM revealed no plastid, bacterial, or mitochondrial contamination in the final preparation.

**Reaction Velocity and Nucleotide Substrate Specificities.** In the presence of 6 mM MgSO₄ and 5 mM ATP, the triphosphatase activity increased linearly with time for up to 20 min (data not shown). The standard incubation time for subsequent characterization studies was chosen as 15 min.

The nuclear NTPase of pea plumules could catalyze the hydrolysis not only of ATP but also of GTP, CTP, and UTP (Table I). However, its activity was not equal with all substrates. The relative efficacy of substrates in promoting NTPase activity was ATP > CTP > GTP > UTP. At 6 mM Mg²⁺, the reaction increased with increasing ATP concentrations, up to 2 mM (Fig. 1). Above 2 mM, the activity became essentially independent of ATP concentration and approached a constant rate. At 1 mM Mg²⁺, concentrations of ATP above 1 mM inhibited ATPase activity (data not shown), so, as is true for other Mg²⁺-dependent ATPases (3), the true substrate for the reaction was Mg²⁺ATP.

Unless otherwise indicated, ATP was used for all other assays to characterize the NTPase. To simplify result reporting, although ATP was the only nucleoside triphosphate used for most assays, the activity assayed will be referred to as a NTPase activity rather than an ATPase activity.

**pH Dependence.** In contrast to the pH optimum of 6.0 to 6.5 usually reported for the K⁺, Mg²⁺-activated ATPases present on the plasma membrane (18), and the optimal pH for the tonoplast ATPases are various from 6.5 to 8.5 (1, 22, 25), the pH optimum for NTPase activity in pea nuclei was around 7.5 (Fig. 2). When experiments were conducted with other substrates (CTP, UTP, and GTP), the pH optimum was still at pH 7.5 in all cases (data not shown).

**Table I. Nucleotide Substrate Specificities**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>NTPase Activity</th>
<th>Relative Activity</th>
<th>ΔP</th>
<th>μmol·min⁻¹·mg⁻¹ protein</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>0.430</td>
<td>100</td>
<td>0.430</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTP</td>
<td>0.289</td>
<td>67</td>
<td>0.289</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>0.240</td>
<td>56</td>
<td>0.240</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTP</td>
<td>0.135</td>
<td>31</td>
<td>0.135</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>0.064</td>
<td>15</td>
<td>0.064</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Tests for Phosphatase Interference. Interference of the assay for NTPase activity by phosphatase activity was avoided in the present study by preincubating nuclei with inhibitors of phosphatases: the acid phosphatase was inhibited by 0.2 mM molybdate (1); the alkaline phosphatase was inhibited by 1.14 mM ascorbic acid (10) (Fig. 2). After their preincubation with inhibitors of phosphatases, the nuclei were tested for phosphatase activity by checking the hydrolysis of ADP which could serve as a substrate for phosphatase but not for NTPase. The amount of released phosphate from ADP was about 15% compared to the amount of released phosphate from ATP, indicating that the phosphatases were strongly inhibited (Table I). Therefore, in the present study, nuclei were preincubated with inhibitors of phosphatases for 20 min at 4°C in all experiments.

Stimulation of ATPase Activity by Divalent Cations. Nuclear NTPase activity was strongly dependent on Mg²⁺. It increased as the concentration of Mg²⁺ increased up to 2 mM, at which concentration it was enhanced several-fold. It remained essentially unchanged between 2 and 6 mM (data not shown). In the presence of 6 mM Mg²⁺, low concentrations of Ca²⁺ stimulated the activity further, with the highest stimulation at 30 to 40 μM Ca²⁺ (Fig. 3). However, within this range of low Ca²⁺ concentrations, Ca²⁺ alone was considerably less effective (Fig. 3). Thus, any significant stimulation of NTPase activity by low concentrations of Ca²⁺ required Mg²⁺. Figure 1 shows the Ca²⁺ stimulation on Mg²⁺-dependent NTPase activity in the presence of varying ATP concentrations. The percentage of stimulation was about 40% at saturating concentrations of the substrate.

Table II summarizes the effect of divalent cations on NTPase activity. Mg²⁺ alone (6 mM) promoted a 14-fold increase in activity, while the same concentration of Ca²⁺ alone only increased the activity 5-fold. The requirement for Mg²⁺ was completely satisfied by Mn²⁺. This is in agreement with the results from studies of other Mg²⁺-dependent ATPases (18, 22). Since MgCl₂ and MgSO₄ activated nuclear NTPase activity to about the same level, and CaCl₂ gave much less stimulation, the activation should be attributed to the divalent cations rather than to the anions.

Effect of Monovalent Cation and Inhibitors. Nuclear NTPase activity was not stimulated by KCl concentrations up to 50 mM at pH 7.5. The effect of KCl was tested both at pH 7.5 and at pH 6.5, which is the optimal pH for both the K⁺-stimulated plasma membrane ATPase (18) and for the Cl⁻-stimulated tonoplast ATPase (6, 22), and there was no stimulation by KCl at either pH (data not shown). These results also indicate that there was little or no plasma membrane or tonoplast contamination in the nuclear preparation.

Table III summarizes the effects of different inhibitors on the NTPase activity of pea nuclei. Among all these inhibitors, the pea nuclear NTPase was sensitive only to quercetin, EGTA, and compound 48/80.

Phytochrome Effects. To test whether phytochrome could modulate ATPase activity in isolated pea nuclei, the effects of R and FR on this activity were investigated. The results (Fig. 4) showed that R significantly increased nuclear ATPase activity and FR reversed the effects of R. Similar results were observed in four consecutive experiments.

Table III. Effect of Inhibitors on NTPase Activity of Pea Nuclei

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Relative Activity</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Vanadate</td>
<td>15 μM</td>
<td></td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>30 μM</td>
<td></td>
<td>116</td>
</tr>
<tr>
<td>Nitrate</td>
<td>50 mM</td>
<td></td>
<td>112</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>2 μg/ml</td>
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<td>106</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.2 mM</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Quercetin</td>
<td>5 μg/ml</td>
<td></td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>10 μg/ml</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>20 μg/ml</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>25 μg/ml</td>
<td></td>
<td>Trace</td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>1 μg/ml</td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>
DISCUSSION

The present study has shown that preparations of nuclei obtained from pea plumes contain a Mg\textsuperscript{2+}-dependent enzyme activity that can catalyze the hydrolysis of GTP, UTP, and CTP in addition to ATP. Because ATPases are found in virtually all plant cell organelles, it was important to clarify whether the NTPass activity we measured in our nuclear preparations was, in fact, nuclear.

Electron microscopic analysis of the nuclear preparation documented the absence of any other recognizable organelles except nuclei. Biochemical characterization of the NTPass activity—specifically analyses of its cation- and anion-dependence, its pH optimum, and its inhibitor sensitivities—distinguished it from other plant ATPases, and thus reinforced the conclusion that the nuclear preparations contained little or no contamination from other organelles.

The pea nuclear NTPass, like the NTPass in animal nuclei, could effectively utilize several nucleoside triphosphates as substrates, although the preference was different: UTP was the least effective nucleoside triphosphate for the pea NTPass, but ranked second (ATP > UTP > GTP) in promoting rat liver nuclear NTPass activity (3, 4). The pH optimum of the NTPass of pea nuclei was around 7.5, similar to that of the nuclear NTPass from rat liver (4). This optimum was the same for all the substrates tested, thus it is unlikely that there was a different NTPass enzyme catalyzing the hydrolysis of each different nucleoside triphosphate.

Most of the nonspecific phosphatase activity in the pH range between 9 and 10 was almost certainly due to alkaline phosphatases. This interference was eliminated by the phosphatase inhibitors, ammonium molybdate and ascorbic acid (Fig. 2). With these inhibitors, at pH 7.5 the enzyme assay was highly specific for NTPass activity.

Plasma membrane ATPase activity is stimulated up to 8 times by K\textsuperscript{+} ions at the optimal concentration of 50 mm (16), and tonoplast ATPase activity is stimulated up to 12 times by Cl\textsuperscript{−} at the same concentration (22). The pea nuclear NTPass was not stimulated by concentrations of KCl up to 50 mm. This clearly distinguishes it from both the plasma membrane and the tonoplast ATPases of other plants.

The inhibitor sensitivity of the pea nuclear NTPass (Table III) was very different from that reported for the plasma membrane ATPase, tonoplast ATPase, or mitochondrial ATPase. At the concentrations tested, vanadate strongly inhibits plant plasma membrane ATPase (23); nitrate blocks tonoplast ATPase (25); ouabain is a selective inhibitor for mitochondrial ATPase (13); but none of them blocks the nuclear ATPase. One of the effective inhibitors, quercetin, also blocks the NTPass activity of rat liver nuclei at this very low micromolar level (3). Quercetin is a flavonoid compound that is commonly found as a secondary plant product in many angiosperms. The possibility that some quercetins possess growth regulatory activities in plants has been raised by several authors. For example, Paliath and Poovaiyah (24) report that quercetin-like flavonoids may act as specific inhibitors of calcium-mediated biochemical processes in plants. Our data would suggest that naturally occurring pea quercetins could serve as inhibitors of nuclear NTPass activity. The possibility of functional interrelationships among calcium, NTPass, and quercetin merits further study.

The relatively selective calmodulin antagonist, compound 48/80 (11), inhibited NTPass activity at as low as 1 \( \mu \text{g} \)/ml. This result indicates that calmodulin, which has been immunocytochemically localized in pea nuclei (28), could be an important regulator of nuclear NTPass activity. Relevant to this hypothesis is the observation that Mg\textsuperscript{2+}-dependent NTPass activity was further stimulated by Ca\textsuperscript{2+} at concentrations as low as 30 \( \mu \text{M} \) for optimal stimulation (Fig. 3). Since the effective concentrations of Ca\textsuperscript{2+} for triggering intracellular physiological responses are also micromolar, this observation is consistent with the postulate that Ca\textsuperscript{2+} may play a role in the intracellular control of NTPass activity.

The presence of phytochrome in the nuclei has been suggested by spectral (9) and immunocytochemical (26) data. Until now the only report of a specific nuclear enzyme activity being regulated by phytochrome was the doctoral thesis of Wagle (31), which describes the stimulation of a Mg\textsuperscript{2+}-dependent ATPase in pea nuclei by the photoactivation of phytochrome in vivo, i.e. before the nuclei were isolated. Figure 4 shows that pea nuclear NTPass activity was photoreversibly modulated by R and FR in vitro. This strongly suggests that functional phytochrome was present in the isolated nuclei. Phytochrome has also been reported to regulate transcription rates (8) and protein phosphorylation (7) in isolated nuclei. The phosphorylation response, like the NTPass stimulation, is also blocked by EGTA and by calmodulin inhibitors (7). Ca\textsuperscript{2+} and calmodulin have been implicated as second messengers for several other phytochrome responses (27).

Matsumoto et al. (20) have reported the activation of ATPase activity in the chromatin fraction of pea nuclei by calcium and calmodulin. Since they did not characterize the pH optimum, nucleotide substrate specificity, inhibitor sensitivity, or light responsiveness of this chromatin-associated enzyme, it would be premature to speculate on whether their enzyme is the same as the NTPass described here. Initial results indicate that most of the NTPass activity in the pea nuclei we isolate is chromatin associated (Y-R Chen, N Datta, SJ Roux, unpublished data), but it remains to be seen whether this fraction is the nuclear NTPass that is light-regulated.

The results presented here have partially characterized a NTPass activity that can be modulated in isolated nuclei by both light and by Ca\textsuperscript{2+}. Given the reported role of NTPass activity in the regulation of RNA processing in animal nuclei, an investigation of whether this enzyme plays a role in the modulation of gene expression by phytochrome in plant nuclei seems warranted.

Acknowledgments—We thank Dr. Neeraj Datta for helpful advice and discussions and Ms. and Mary Miller for her electron microscopic analyses of purified nuclei.

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