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Noncompetitive Inhibition of Plant Protein Ser/Thr Phosphatase PP7 by Phosphate

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Changes in the cytoplasmic inorganic phosphate (P_i) concentrations are an important cue for the plant cells to regulate their metabolism and phosphate homeostasis. However, phosphate sensors/receptors involved in this regulation are largely unknown. P_i is a common nonspecific competitive inhibitor of phosphatases, usually in millimolar range. Here we report a procedure to refold recombinant *Arabidopsis thaliana* protein Ser/Thr phosphatase PP7 and demonstrate that PP7 is inhibited by submillimolar P_i concentrations (IC_{50} = 0.66 ± 0.14 mM) via a mainly noncompetitive mechanism. The results indicate that PP7 may possess a specific P_i-binding site responsible for its allosteric regulation, and suggest a possible phosphate sensor function for this protein phosphatase. © 2001 Academic Press

Key Words: **protein phosphorylation; protein Ser/Thr phosphatase; PP7; regulation; inorganic phosphate; phosphate homeostasis; *Arabidopsis thaliana*.**

Plant cells are able to detect changes in cytoplasmic P_i concentrations and adjust accordingly P_i uptake and its release from the vacuole into the cytoplasm (1, 2). Both long-term (e.g., transcriptional activation of phosphate transporters) and short-term (e.g., rapid up-regulation of P_i uptake) responses of plant cells to phosphate depletion have been documented (1, 2). However, the phosphate sensors and the signaling pathways involved in these responses are largely unknown.

We have recently identified novel plant protein Ser/Thr phosphatases, termed PP7 (3), which are members of the PPP family (4) and are structurally more closely related to the novel rdgC/PP5 subfamily than to the "classical" PP1, PP2A, or calcineurin subfamilies (5, 6). Plant PP7 have no known close homologs in other kingdoms and are likely to be conserved at least between mosses and higher plants (7). While the presence of the PP7 transcript is detectable in all organs in *Arabidopsis thaliana*, *in situ* hybridization indicates the highest expression levels in a subset of stomata, which is suggestive of a role in sensory signaling (8). Soluble PP7 expressed in *E. coli* is mainly a proteolyzed product cleaved by an endogeneous *E. coli* protease at the insert in the middle of its catalytic domain (the first and the longest of the three inserts found in PP7). Biochemical characterization of this PP7 form has demonstrated that the recombinant protein is an active protein Ser/Thr phosphatase resistant to okadaic acid and calyculin A, and suggested that the first insert may play role of an autoinhibitory region, possibly through a pseudosubstrate site (9).

In this report, we describe a procedure to refold the entire recombinant PP7, and demonstrate that its activity is noncompetitively inhibited by submillimolar concentrations of inorganic phosphate. These observations indicate that P_i may be a specific allosteric regulator of PP7 and suggest a possible role of phosphate sensor for this protein phosphatase.

EXPERIMENTAL PROCEDURES

Heterologous PP7 expression and phosphatase assays. Assays were performed as described previously (9).

PP7 refolding by dilution. PP7 was purified from the inclusion bodies under denaturing conditions according to Qiagen instructions with two minor modifications: IMAC chromatography was performed on a Ni^{2+}-iminodiacetate Sepharose column and PP7 was eluted with 250 mM imidazole (pH 7.5), 8 M urea and supplemented with 0.1 M dithiotreitol (DTT). For refolding, PP7 solution (0.3–1 μM) was mixed by vortexing with 200-fold excess of the refolding buffer containing 40 mM Tris–HCl (pH 7.5), 60 mM NaCl, 0.2 mM EDTA, 1 mM benzamidine and 1.5 μM bovine serum albumin. In a standard procedure, the refolding mixture was allowed to stand for 1 h at room

Abbreviations used: DTT, dithiotreitol; *p*NPP, *p*-nitrophenylphosphate; PAGE, polyacrylamide gel electrophoresis.

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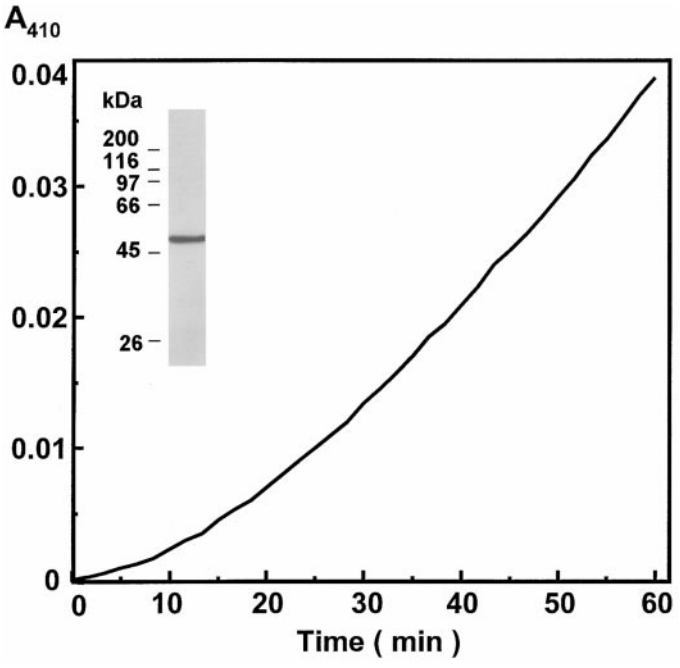

FIG. 1. Time course of PP7 renaturation. PP7 purified from inclusion bodies by Ni^{2+}-iminodiacetate Sepharose chromatography (insert) was refolded as described under Experimental Procedures (final concentration 20 nM). $MnCl_2$ (0.5 mM) and *p*NPP (2 mM) were added immediately after dilution, and PP7 refolding followed as an increase in *p*-nitrophenol absorption (A_{410}).

temperature, after which $MnCl_2$ was added to a final concentration 0.5 mM. In some experiments, $MnCl_2$ and *p*-nitrophenylphosphate (*p*NPP) were added immediately after dilution. Refolded PP7 was kept on ice and used within several hours.

RESULTS

PP7 refolding. As we have previously reported (9), His_6-tagged *A. thaliana* PP7 expressed in *E. coli* is found mainly in the inclusion bodies and can be purified by standard IMAC chromatography under denaturing conditions. Purified PP7 with expected molecular weight ~50 kDa (Fig. 1, insert) can be refolded by two-step dialysis, but no detectable phosphatase activity is associated with the refolded protein (9). In this work, we have tested an alternative refolding method by rapid dilution. Urea-solubilized PP7 purified from the inclusion bodies was found to represent disulfide-linked oligomers appearing as a ladder when analyzed by SDS–PAGE without reducing agents (not shown). Therefore, a reduction step with 0.1M DTT was introduced prior to PP7 refolding. Refolding by rapid dilution was found to yield an active protein (Fig. 1) with a specific activity with *p*-nitrophenylphosphate (*p*NPP) 10–20% of the activity of the soluble PP7 described previously (9), which is cleaved by an endogeneous *E. coli* protease within the first of the three inserts found in the PP7 catalytic domain and thus consists of two fragments of 25 and 28 kDa. Lower specific activity of the refolded PP7 can probably be attributed to the presence of the intact first insert, which is likely to act as an autoinhibitory region (9). Although some dephosphorylation of myelin basic protein (the best protein substrate of the cleaved PP7 (9)) could be detected upon prolonged incubation, the activity of the entire PP7 with this substrate was too low to be accurately measured over shorter incubation times necessary to insure linearity. The experiments described below were therefore done using *p*NPP as PP7 substrate.

The presence of DTT (3–5 mM) in the refolding buffer was found to be required for successful refolding (as judged from specific activity). However, this effect of DTT was not due to its properties of a reducing agent, but probably to its ability to chelate traces of inhibitory metals, as DTT could be replaced with submillimolar concentrations of EDTA. This observation is similar to that by Zhuo and Dixon (10) for a phosphatase from bacteriophage λ, which is related to the PPP family. The refolding step sensitive to the presumed inhibitory metal(s) was apparently very rapid, since EDTA or DTT were efficient only when initially present in the dilution buffer but not when added 15 s after protein dilution or later (data not shown). The phosphatase activity gradually increased during 30–40 min after dilution and then stabilized (Fig. 1). Therefore, in a standard refolding procedure, 1 h refolding time was allowed. The activity was stable for several hours and then gradually declined. No activity could be detected after 36–48 h storage at +4–8°C, i.e., conditions used for refolding by dialysis, which possibly accounts for the failure of the latter method to yield active PP7.

Like cleaved PP7 (9), refolded entire PP7 required Mn^{2+} for its activity (EC_{50} ~30 μM; data not shown). Therefore, after the completion of refolding (~1 h), EDTA was neutralized by adding excess Mn^{2+}.

Noncompetitive inhibition of PP7 by inorganic phosphate. PP7 was found to be highly sensitive to inhibition by inorganic phosphate, a common competitive inhibitor of phosphatases (Fig. 2A; $IC_{50} = 0.66 \pm 0.14$ mM, mean ± standard deviation, n = 13). IC_{50} did not change significantly in the presence of increased (1.5 mM) $MnCl_2$ concentration (not shown). Surprisingly, further analysis revealed not only an expected increase in K_m (from to 1.7 mM to ~3 mM), but also a dramatic decrease in V_{max} (from 0.15 to 0.03 μM pNPP · min^{-1}) with the phosphate concentrations increasing from 0 to 6 mM (Fig. 2B). Thus, P_i acts in this range of concentrations also as a noncompetitive inhibitor, suggesting that the regulation may be allosteric.

DISCUSSION

The conservation of PP7 between mono- and dicotyledons and the detection of PP7-related cDNA frag-

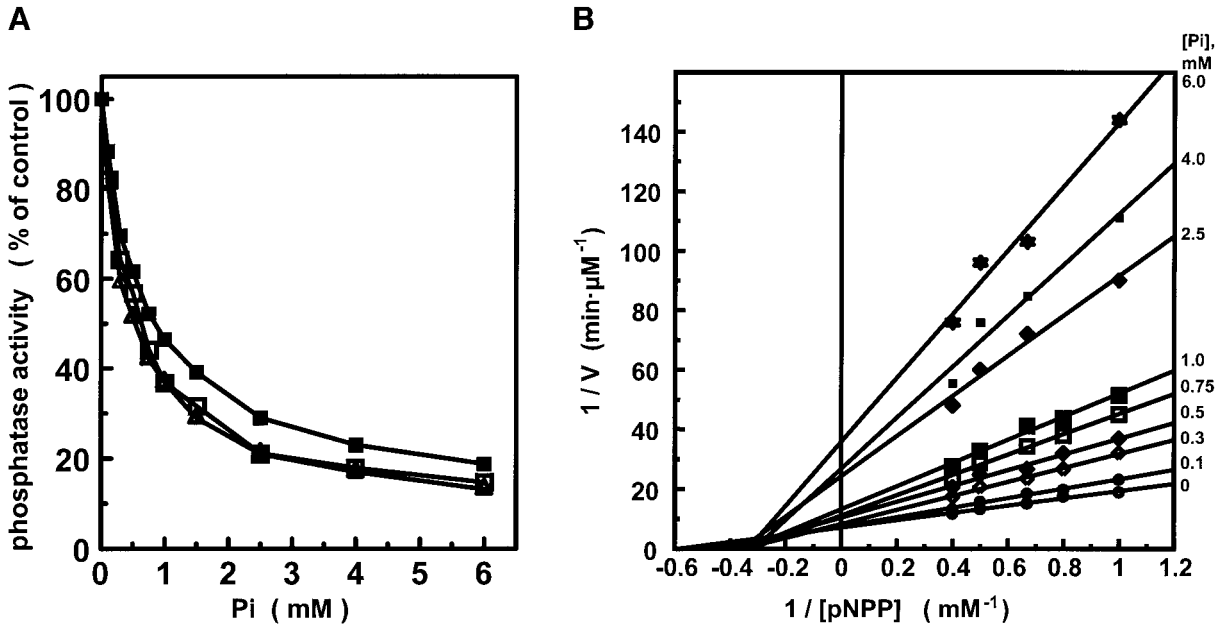

FIG. 2. Inhibition of recombinant PP7 by inorganic phosphate. (A) The effect of P_i on the activity of refolded PP7 (20 nM) at 1 mM (empty triangles), 2 mM (empty squares), and 3 mM (filled squares) *p*NPP. (B) A Lineweaver–Burk double-reciprocal plot of PP7 activity in the presence and absence of increasing concentrations of P_i as indicated.

ments in a moss *Physcomitrella patens* (5, 7) indicate that these protein phosphatases may have an essential function in land plants. However, PP7 contains no recognizable regulatory domains, and its primary structure alone provides no clues to its possible functions and regulation.

Biochemical characterization of recombinant proteins proved useful for understanding of the regulation mechanisms of other members of the PPP family of protein phosphatases. Despite significant sequence similarity (≥35% identity) between different members of this family and probably common principles of their 3-dimensional organization (11), the attempts to express them in heterelogous systems have met variable success. While some PPP phosphatases can be obtained in a soluble active form or easily refolded (e.g., PP1 (12), calcineurin (13, 14)), the functional expression of others may require years of work (e.g., PP2A (15)).

We have previously expressed in *E. coli* and biochemically characterized *A. thaliana* PP7 (9). However, the soluble active PP7 was found to be cleaved in two halves by an endogeneous *E. coli* protease, which potentially limits its suitability for further analysis. While our initial attempts to refold entire *A. thaliana* PP7 yielded soluble but inactive protein (9), in this report we succeeded in obtaining the active entire PP7 suitable for further characterization.

Inorganic phosphate is a common competitive inhibitor of protein phosphatases with typical IC_{50} values in the millimolar range (16–20). It was therefore surprising to find that P_i inhibits PP7 in submillimolar concentrations in a partially noncompetitive manner. This suggests that PP7 is likely to possess a phosphate-binding site responsible for its allosteric regulation. Estimations of the *in vivo* cytoplasmic P_i concentrations in plant cells (derived mainly from ^{31}P-NMR spectrometry studies) vary significantly and range from few millimolar to above 20 mM ((1) and references therein). However, the term "cytoplasmic" is usually employed as synonymous with "nonvacuolar." The actual distribution of P_i within the cytoplasm and between the cytoplasm and different organelles is unknown. It has been argued that at least in *Chlamydomonas* most of the ^{31}P-NMR signal of inorganic phosphate originates from the chloroplast (21). If the chloroplasts accumulate disproportionally high concentrations of P_i in higher plants as well, the real free phosphate concentrations in the cytoplasm are likely to be in the low millimolar or even submillimolar range, close to the range of PP7 regulation observed in our work. This suggests that P_i may be a genuine PP7 regulator *in vivo*.

Changes in the cytoplasmic P_i concentration are probably an important cue for the plant cells to regulate their metabolism and phosphate homeostasis (1, 2). There is at least one described example of a physiologically important regulation of a plant protein phosphatase by intracellular P_i. A decrease in the cytoplasmic P_i concentration in the mesophyll cells in the light is thought to be essential for dephosphorylation and activation of sucrose phosphate synthase by PP2A (22, 23). The sensitivity of PP2A to the inhibition by P_i increases in the dark with a dramatic IC_{50} shift from 11 to 2 mM. While in the low-affinity state P_i acts as a

competitive inhibitor (as expected for any protein phosphatase), in the high-affinity state the inhibition is by a mixed mechanism affecting both K_m and V_{max}. The molecular basis of this regulation is not known but appears to require protein synthesis (23) and may therefore rely on an additional phosphate-sensing regulatory protein which mediates the allosteric regulation. In the case of PP7, the catalytic subunit itself appears to be sufficient for a similar regulation.

On the basis of these data, it is tempting to speculate that PP7 might be one of the phosphate sensors and function to translate the fluctuations of the phosphate levels into the changes in gene expression or to directly dephosphorylate proteins involved in P_i homeostasis. *In vivo* studies using transgenic plants with altered PP7 expression should allow testing these hypotheses.

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