

Cloning, Expression in *Escherichia coli*, and Characterization of *Arabidopsis thaliana* UMP/CMP Kinase¹

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A cDNA encoding the *Arabidopsis thaliana* uridine 5'-monophosphate (UMP)/cytidine 5'-monophosphate (CMP) kinase was isolated by complementation of a *Saccharomyces cerevisiae* *ura6* mutant. The deduced amino acid sequence of the plant UMP/CMP kinase has 50% identity with other eukaryotic UMP/CMP kinase proteins. The cDNA was subcloned into pGEX-4T-3 and expressed as a glutathione S-transferase fusion protein in *Escherichia coli*. Following proteolytic digestion, the plant UMP/CMP kinase was purified and analyzed for its structural and kinetic properties. The mass, N-terminal sequence, and total amino acid composition agreed with the sequence and composition predicted from the cDNA sequence. Kinetic analysis revealed that the UMP/CMP kinase preferentially uses ATP (Michaelis constant [K_m] = 29 μM when UMP is the other substrate and K_m = 292 μM when CMP is the other substrate) as a phosphate donor. However, both UMP (K_m = 153 μM) and CMP (K_m = 266 μM) were equally acceptable as the phosphate acceptor. The optimal pH for the enzyme is 6.5. P^1 , P^5 -di(adenosine-5') pentaphosphate was found to be a competitive inhibitor of both ATP and UMP.

Pyrimidines are intimately involved in the physiology of cells. They participate at multiple levels in intermediary and secondary metabolism from nucleotide and macromolecule biosynthesis to the biosynthesis of complex carbohydrates and the metabolic regulation of intermediary metabolism. All pyrimidines within the cell are derived from UMP, which arises either from the de novo pyrimidine biosynthetic pathway or from salvage of preformed pyrimidines. UMP/CMP kinase converts uridine and cytidine monophosphates into the corresponding uridine and cytidine diphosphates. Because all pyrimidines are derived from UMP, UMP kinase is the first committed step and one of the central enzymes in the further anabolism of pyrimidine nucleotides.

The importance of pyrimidine monophosphokinases to cell physiology has been firmly established. In both bacteria and yeast, the roles of pyrimidine monophosphokinases with respect to cell proliferation and physiology have been widely studied. In *Escherichia coli*, the product of the UMP kinase gene (*pyrH/smbA*) has been shown to influence cell

proliferation. Yamanaka et al. (1992), working with the *mukB* gene, isolated a suppressor of *mukB* that they termed *smbA*. The *smbA* phenotype is pleiotropic. First, the *smbA* mutant ceased macromolecular synthesis, was hypersensitive to SDS, and showed a novel morphological phenotype under nonpermissive conditions. Later, it was found that the wild-type *smbA* gene is identical to the *pyrH* gene and that the *smbA2* mutant protein encodes an unstable UMP kinase with impaired catalytic and regulatory functions.

In yeast, mutations in the UMP kinase gene have been shown to cause a conditional lethal phenotype (Liljelund and Lacroute, 1986). When grown at the nonpermissive temperature, the UTP and CTP pools decline to 10% of their wild-type levels, which affects both RNA and protein synthesis and ultimately results in cell death. Complementation of this mutant permitted the first isolation of a eukaryotic UMP kinase gene (Liljelund and Lacroute, 1986). This is the same mutant that we have complemented in this study to isolate the *Arabidopsis thaliana* UMP/CMP kinase cDNA.

Other pyrimidine monophosphokinases are also important in normal cellular physiology. The *cdc8* mutant of *Saccharomyces cerevisiae* was isolated as a cell-cycle-deficient mutant that was defective in nuclear division (Newlon and Fangman, 1975). When incubated at the restrictive temperature, the *cdc8* mutant cells arrest in S phase with a typical dumbbell morphology. This mutation also produces other pleiotropic effects, including inhibition of normal cellular DNA replication (Birkenmeyer et al., 1984), involvement in error-prone repair (Prakash et al., 1979; Baranowska and Zuk, 1991), and involvement in repair of single-stranded breaks (Baranowska et al., 1990). The *CDC8* gene was isolated (Birkenmeyer et al., 1984; Kuo and Campbell, 1983) and found to encode dTMP kinase (Jong et al., 1984). Known suppressors of the *CDC8* gene have also been isolated and characterized. One of these, *SOC8*, encodes the UMP kinase gene *ura6* (Jong et al., 1993). Thus, nucleotide monophosphokinases can have profound effects on cellular morphology and physiology.

Substrate utilization by eukaryotic UMP kinases has also received much study (Weismüller et al., 1990; Jong et al., 1993; Müller-Dieckmann and Schultz, 1994). Whereas all

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Abbreviations: Ap_5A , P^1 , P^5 -di(adenosine-5') pentaphosphate; GST, glutathione S-transferase; IPTG, isopropyl- β -D-thiogalactoside.

eukaryotic enzymes have specificity for both UMP and CMP, the yeast enzyme also has specificity for AMP. Indeed, the yeast UMP kinase has such a high affinity for AMP that the *ura6* gene has been isolated as a multicopy suppressor of yeasts deficient in adenylate kinase (Schricker et al., 1992). The yeast UMP kinase gene also complements the *E. coli* adenylate kinase enzyme. In contrast, the cellular slime mold (*Dictyostelium discoideum*) enzyme does not complement either the yeast or the *E. coli* adenylate kinase mutants. UMP kinase is also required for the metabolic activation of several important anti-tumor drugs, including 5-fluorouracil and Ara-C (Seagrave and Reyes, 1987). Consequently, UMP kinase plays a central and very important role in pyrimidine anabolism.

In addition to studies of the eukaryotic enzyme, the prokaryotic enzyme has received much attention (Valentin-Hansen, 1978; Yamanaka et al., 1992; Serina et al., 1995, 1996). Because of this, significant differences between the enzymatic activities of the prokaryotic and eukaryotic enzymes have been identified. The prokaryotic enzyme is allosterically regulated by both GTP and UTP (Serina et al., 1995). GTP functions to stimulate the enzyme when there is an overabundance of purine triphosphates, and UTP down-regulates the enzyme when pyrimidine triphosphates have accumulated to a high level. In addition, UMP kinase is further regulated by divalent metal ions in a novel mechanism related to metal-free-UTP binding (Serina et al., 1996). The binding of metal-free UTP causes a gel-sol transition that affects the state of UMP kinase aggregation and, subsequently, the enzyme activity.

In humans, UMP kinase is associated with an autoimmune deficiency that results in susceptibility to respiratory infections such as invasive *Hemophilus influenzae* type B disease in Alaskan Eskimos (Petersen et al., 1985) and South American Indians (Gallango and Suinaga, 1978; Gallango et al., 1978). This underexpression of UMP kinase results in a syndrome similar to the immune defect resulting from adenosine deaminase deficiency (Giblett et al., 1974), which is thought to be due to the toxic build-up of substrates.

In plants, UMP kinase has received only marginal study. UMP kinase is elevated during seedling development (Deng and Ives, 1972; Mazur and Buchowicz, 1972) and fruit ontogeny (Rudd and Fites, 1972; Deng and Ives, 1975). Because UMP kinase is likely to be as important in plants as it is in microorganisms, we have isolated the cDNA for the *A. thaliana* UMP/CMP kinase, expressed the coding region in *E. coli*, and characterized the resulting plant enzyme.

MATERIALS AND METHODS

The pGEX-4T-3 and glutathione-Sepharose 4B were purchased from Pharmacia. The vector pT7-Blue was from Novagen (Madison, WI). Enterokinase was from Biozyme Laboratories (San Diego, CA). Restriction enzymes, T4 ligase, and *Taq* polymerase were from Promega. All other enzymes and reagents were obtained from Sigma unless otherwise noted. The *Arabidopsis thaliana* cDNA library in a yeast transformation vector was previously described (Mi-

net et al., 1992). Oligonucleotides were synthesized at the Iowa State University (Ames) Nucleic Acid Facility.

Strains

Saccharomyces cerevisiae FL100a was the wild-type strain used for all yeast-related manipulations. The *S. cerevisiae* *ura6* strain was derived from FL100a and displayed a conditional thermosensitive and 5-fluorouracil-resistant phenotype (Liljelund and Lacroute, 1986). The strain used in these studies was also auxotrophic for His and Trp. Genotypes of the yeast strains were determined by plating at either the permissive or restrictive temperature on media lacking various ingredients. The *Escherichia coli* strain XL1-Blue was used for all bacterial manipulations.

Yeast Methods

Yeast transformation was conducted as described previously (Gietz et al., 1992). *S. cerevisiae* auxotrophic mutants were complemented by *A. thaliana* cDNAs as previously described (Minet et al., 1992). To rescue the plasmids from yeast, a loopful of yeast cells was suspended in 0.4 mL of 10 mM Tris, pH 8.0, 1 mM EDTA, and 0.4 M NaCl and vortexed (2 min) with 200 μ L of glass beads. Following vortexing, an equal volume of phenol/chloroform (1:1) was added and the vortexing was repeated. After the sample was centrifuged, 250 μ L of supernatant was removed and DNA was precipitated by the addition of 500 μ L of ethanol. The sample was washed with 70% ethanol, and the DNA was resuspended in water and used for electroporation of *E. coli* (Ausubel et al., 1993).

Recombinant DNA Methods

DNA-sequencing reactions were performed using the Prism Dye-Deoxy cycle sequencing kit (Applied Biosystems). The reactions were run on a DNA sequencer (Prism 377, Perkin-Elmer). Sequencing was initiated from known vector sequences. On the basis of these runs, oligonucleotide primers specific to the *A. thaliana* UMP/CMP kinase gene sequence were constructed. Both strands were completely sequenced.

The 606-bp UMP kinase-coding region was PCR amplified from the pAt-URA6 clone using a pair of oligonucleotides, LZ2080 (5'-GCGGATCCGATGACGATGACAAG-ATGGGATCTGTTGATGCTGCT-3') and LZ2081 (5'-CG-GCTCGAGCTACTAGGCTTCAACCTTCTCAGC-3'). The PCR product was cloned into the pT7-Blue(R) T-vector to form the vector pRT379. These oligonucleotide primers introduced into the PCR produced unique *Bam*HI and *Xho*I sites at the ends of the PCR fragment, an enterokinase site for cleavage of the GST fusion protein, and an additional stop codon. Following cloning of the PCR product in pRT379, the sequence of the coding region was confirmed by completely sequencing the insert. The expression vector pRT380 was subsequently prepared by inserting the 637-bp *Bam*HI/*Xho*I fragment from pRT379 into the *Bam*HI/*Xho*I sites of pGEX-4T-3. Again, the sequence of the coding region was confirmed by completely sequencing the insert.

Expression in *E. coli*

The vector pRT380 was transformed into *E. coli* XL1-Blue for the production of the fusion protein. To induce the fusion protein, 5 mL of overnight culture was diluted into 500 mL of 2YT medium (1.6% bactotryptone, 1.0% yeast extract, and 0.5% NaCl, pH 6.0) and grown at 37°C until mid-log phase. IPTG was then added to a final concentration of 1 mM. Growth continued for 3 h at 37°C. Cells were harvested by centrifugation at 4000g for 10 min. The cell pellet was stable when stored at -20°C.

Purification of UMP/CMP Kinase

Frozen cells were thawed in 10 mL of PBS containing 25 mg of lysozyme. After 30 min at room temperature, the cells were sonicated on ice. DNase I (2 mg) in 0.8 M MgCl₂ was added and the cell sonicate was incubated at room temperature for 10 min. Cellular debris were removed by centrifugation at 12,000g for 40 min, and the supernatant was used to resuspend 0.3 mL of packed PBS-washed glutathione-Sepharose beads. The beads and supernatant were incubated overnight at room temperature on a rotating wheel. The beads were removed by centrifugation and washed with PBS until the supernatant was clear (usually five times). The fusion protein could be eluted by the addition of 10 mM glutathione. However, for most studies, the UMP/CMP kinase domain was removed from the bound fusion protein by resuspending the beads in 0.35 mL of enterokinase buffer (25 mM Tris, pH 7.5, and 10 mM CaCl₂) and digesting with 1000 units of enterokinase until completion at room temperature on a rotating wheel.

After digestion, the supernatant was recovered by centrifugation and applied to a reverse-phase C₁₈ HPLC column (250 × 10 mm, i.d.; Vydac, Hesperia, CA). The column was eluted at a rate of 4 mL min⁻¹ with a programmed elution profile. Solvent A was 0.1% trifluoroacetic acid in water and solvent B was 0.08% trifluoroacetic acid in acetonitrile. The enzyme was eluted by a series of linear gradients: 0 to 5 min, gradient from 20 to 40% solvent B; 5 to 23 min, gradient from 40 to 43% solvent B; the column was cleaned and equilibrated for further separations by two gradients: 23 to 26 min, gradient from 43 to 100% solvent B; and 26 to 28 min, gradient back to 20% solvent B. The UMP/CMP kinase was eluted at 41% solvent B. Following elution, the enzyme fraction was dialyzed against 20 mM Mes, pH 6.5, at 4°C for 24 h and then concentrated to about 0.5 mL using a centrifugal concentrator (Centricon-10, Amicon, Beverly, MA).

Protein Methods

Protein concentration was determined by the method of Bradford (1976) with BSA as a standard. SDS-PAGE was performed according to the method of Laemmli (1970). N-terminal amino acid analysis was performed in the Iowa State University Protein Facility (Ames) by sequential Edman degradation on a protein sequencer (model 477A, Applied Biosystems) and a protein analyzer (model 120A, Applied Biosystems). Amino acid composition was per-

formed with phenyl isothiocyanate-derivatized amino acids following hydrolysis of purified UMP/CMP kinase in 6 N HCl.

Matrix-assisted laser-desorption ionization MS was used for determining molecular mass. Protein samples of 0.5 to 1.0 μL containing about 0.5 to 1 μg of protein were loaded with 0.5 μL of freshly prepared 3,5-dimethoxy-4-hydroxy cinnamic acid matrix onto a time-of-flight mass analyzer (Lasermat 2000 MALDI, Finnigan, Madison, WI). The collected data were analyzed using data processing software (Lasermat 2000, Finnigan). Lysozyme was used as an internal calibration standard.

Enzymatic activity was determined spectrophotometrically by measuring the formation of ADP and UDP at 23°C with a coupled-enzyme assay (Agarwal et al., 1978). Quantitation was performed by following the decrease of NADH A₃₄₀. The initial-rate data were analyzed for kinetic mechanisms (Fromm, 1975) by using a computer program written in the MINITAB language with an α-value of 2.0 (Siano et al., 1975).

RESULTS

Cloning by Complementation

Yeast genetic crosses were made between *ura2*⁻ and *ura6-15*^(Ts) to provide progeny blocked in de novo pyrimidine biosynthesis as well as in the conversion of UMP into UDP. The inclusion of the *ura2*⁻ mutation provided a better screen for *ura6-15*^(Ts). These progeny were unable to make pyrimidines via the de novo pyrimidine biosynthetic pathway, but could be rescued at the permissive temperature by the addition of uracil through the salvage pathway. Strain *ura6-15A*, which has an FL100a, *ura6-15*^{Ts}, *ura2*⁻, *trp*⁻, *his*⁻ genotype, was used for all transformation experiments.

The yeast strain was transformed with an Arabidopsis cDNA library in the vector pFL61 (Minet et al., 1992). Approximately 54,000 individual transformants were screened for colonies at the restrictive temperature. Two colonies were found from the 54,000 transformants. DNA was isolated from each of these yeast strains and *E. coli* was transformed by electroporation. Both colonies yielded the identical cDNA clone when analyzed by DNA sequence analysis. After growth in *E. coli*, both clones were reisolated and used to transform the original *ura6-15A* strain to verify that the clones produced the URA⁺ phenotype. The plasmid containing this clone was termed pAt-*ura6*.

Analysis of the UMP/CMP Kinase mRNA

The DNA sequence of the pAt-*ura6* insert is presented in Figure 1. This presumptive Arabidopsis UMP/CMP kinase cDNA sequence has been deposited in GenBank as accession no. AF000147 and is 895 nucleotides long. It shows a 60-nucleotide 5'-untranslated region, a coding region of 606 nucleotides, and a 175-nucleotide 3'-untranslated region extending to the poly(A⁺) site at position 874. This cDNA did not show a typical polyadenylation signal. The closest to the consensus is AATTTT, which is duplicated at

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OCAAATCTCC TCCGNGPTTC CTTCGCTCC AGGAGATATT TTAACAAGT TCTTAGTACA      60
5          10          15          20
ATG GGA TCT GTP GAT GCT GCT AAT GGA AGT GGG AAG AAA CCT ACA GTT ATA TTT GTT CTT
Met Gly Ser Val Asp Ala Ala Asn Gly Ser Gly Lys Lys Pro Thr Val Ile Phe Val Leu      120
25          30          35          40
GGT GGT CCA GGA AGT GGA AAA GGT ACC CAG TGT GCT TAT ATT GTT GAA CAT TAT GGT TAC
Gly Gly Pro Gly Ser Gly Lys Gly Thr Gln Cys Ala Tyr Ile Val Glu His Tyr Gly Tyr      180
45          50          55          60
ACA CAT CTG AGT GGT GGA GAT CTT CTT AGA GCT GAG ATT AAA TCA GGT TCT GAA AAT GGA
Thr His Leu Ser Ala Gly Asp Leu Leu Arg Ala Glu Ile Lys Ser Gly Ser Glu Asn Gly      240
65          70          75          80
ACT ATG ATC CAG AAT ATG ATT AAA GAG GGG AAG ATT GTA CCT TCT GAG GAT ACT ATC AAG
Thr Met Ile Gln Asn Met Ile Lys Glu Gly Lys Ile Val Pro Ser Glu Val Thr Ile Lys      300
85          90          95          100
CTT CTA CAG AAA GCT AIT CAG GAA AAC GGG AAT GAC AAG TTC CTC ATT GAT GGT TTC CCT
Leu Leu Gln Lys Ala Ile Gln Glu Asn Gly Asn Asp Lys Phe Leu Ile Asp Gly Phe Pro      360
105          110          115          120
CGT AAT CAG GAA AAC CGA GCA CCA TTT GAA AAA GTT ACT CAG ATT GAA CCA AAG TTT GTC
Arg Asn Glu Glu Asn Arg Ala Ala Phe Glu Lys Val Thr Glu Ile Glu Pro Lys Phe Val      420
125          130          135          140
TTA TTC TTC GAT TGT CCT GAG GAA GAG ATG GAG AAG CGC CTG TTG GGC CGA AAC CAG GGG
Leu Phe Phe Asp Cys Pro Glu Glu Glu Met Glu Lys Arg Leu Leu Gly Arg Asn Gln Gly      480
145          150          155          160
AGA GAG GAT GAC AAT ATT GAG ACT ATA AGG AAG CGC TTT AAG GTG TTT CTT GAA TCT AGC
Arg Glu Asp Asp Asn Ile Glu Thr Ile Arg Lys Arg Phe Lys Val Phe Leu Glu Ser Ser      540
165          170          175          180
TTA CCA GTG ATT CAT TAC TAC GAA GCT AAG GGG AAA GPT AGG AAG ATT AAT GCT GCA AAG
Leu Phe Val Ile His Tyr Tyr Glu Ala Lys Gly Lys Val Arg Lys Ile Asn Ala Ala Lys      600
185          190          195          200
CCC ATT GAA GCT CTC TTC GAG GAG GTG AAG GCA ATT TTT TCT CCT GAA GCT GAG AAG GPT
Pro Ile Glu Ala Val Phe Glu Glu Val Lys Arg Ala Ile Phe Ser Pro Glu Ala Glu Lys Val      660
202
GAA GCC TAG      669
Glu Ala ***

GCTGCGACAT CGPAAAAAGA TACCGAARAC CGGATAAATT GATCAGGTAG AGTTCGGTGT      729
GTGCACTCTT TCTTACAGGA AGTCTCTGCT TGGCCGATTT TGTTTTTTTG CTTATTAACC      789
ATATGATGTA TTACTGCTAT TATTATACCA AATTACCTA TTATATAATT TTCTTTTCTC      849
TTCAATTTTT GTATTGATG GSATTAATAA AAAAAAATAA AAAAAA      895

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Figure 1. cDNA sequence of the *A. thaliana* UMP/CMP kinase. The nucleotide number is presented at the end of each row. The deduced amino acid sequence is presented below the DNA sequence and the amino acids are numbered above the DNA sequence. Asterisks indicate the amber (UAG) codon.

positions 836 to 841 and 853 to 858. DNA matrix analysis revealed no significant regions of internal duplication or inverted repeats within the cDNA sequence.

The translation start codon correlates well with the rules of Kozak (1986). The sequence at the translation start differs from the eukaryotic consensus at only a single nucleotide (ACAATGG). Consequently, this cDNA is expected to be translated very efficiently.

Analysis of the UMP/CMP Kinase Protein

The deduced amino acid sequence of the Arabidopsis UMP/CMP kinase is also presented in Figure 1. The protein is 202 amino acids long. There is a pair of conserved Cys residues that are shared among all of the eukaryotic UMP kinases. However, it has been proposed that the conserved Cys residue found at position 31 in the Arabidopsis protein has a free SH group (Weismüller et al., 1990).

The Arabidopsis UMP/CMP kinase amino acid sequence is very similar to UMP kinases from other eukaryotic sources (Fig. 2). UMP kinases have been isolated from yeast (*S. cerevisiae*), a cellular slime mold (*D. discoideum*), and a mammal (*Sus scrofa*). Each of these enzymes shows 47 to 53% amino acid identity with each other. It was interesting that 32.2% of the amino acid residues are identical among all of the four proteins. If conservative substitutions are permitted, then the homology among the four proteins increases to 47.5%.

There is a conserved region near the N terminus that is maintained in nucleotide-binding proteins (Möller and Amons, 1985). This sequence, GGPGS/A GK, is also preserved in the eukaryotic nucleotide monophosphokinases. In crystallographic studies of adenylate kinase, Pai et al. (1977) found that this loop anchors the γ -phosphate moiety of ATP.

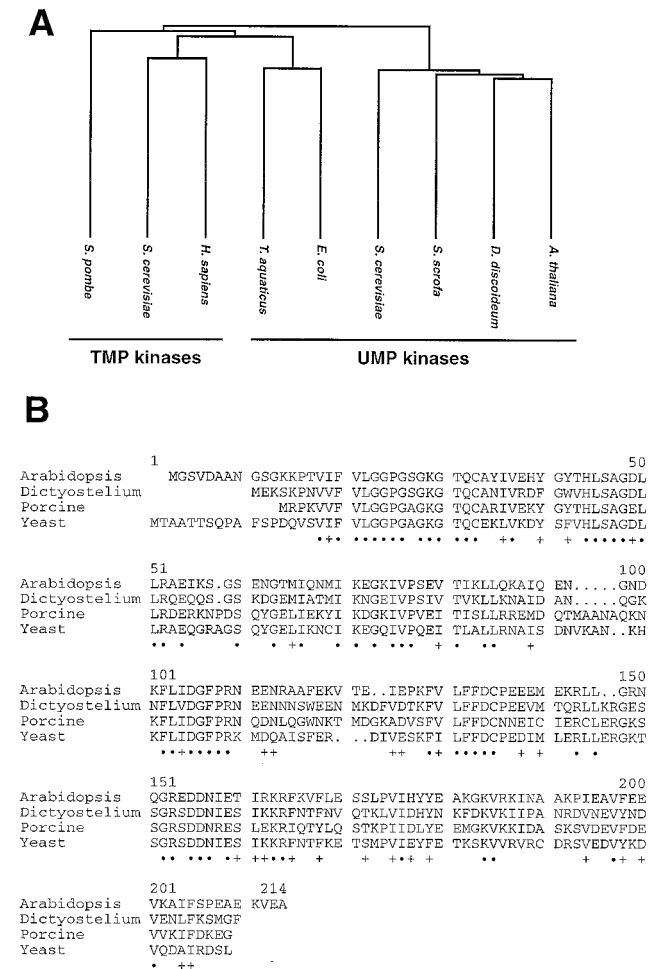


Figure 2. Comparison of pyrimidine monophosphokinases. A, Comparison of the amino acid identity between the various pyrimidine monophosphate kinases was performed using the PileUp program from the Genetics Computer Group (Madison, WI). For this analysis, both UMP kinases and TMP kinases were included. UMP kinases were included from archeobacterial, eubacterial, and eukaryotic (plant, mammal, yeast, and mold) sources. The GenBank accession numbers for the sequences used in this study are as follows: *Saccharomyces pombe* TMP kinase, L04126; *S. cerevisiae* TMP kinase, K02116; *Homo sapiens* TMP kinase, L16991; *Thermus aquaticus* UMP kinase, X83598; *E. coli* UMP kinase, X78809; *S. cerevisiae* UMP kinase, M69295; *S. scrofa* UMP kinase, D29655; *D. discoideum* UMP kinase, M34568; and *A. thaliana* UMP/CMP kinase, AF000147. B, Alignment of amino acid sequences of eukaryotic UMP kinases from a plant (*A. thaliana*), a mammal (*S. scrofa*), a yeast (*S. cerevisiae*), and a cellular slime mold (*D. discoideum*). Identical residues are marked by dots (•) and conservative substitutions are indicated with plus signs (+).

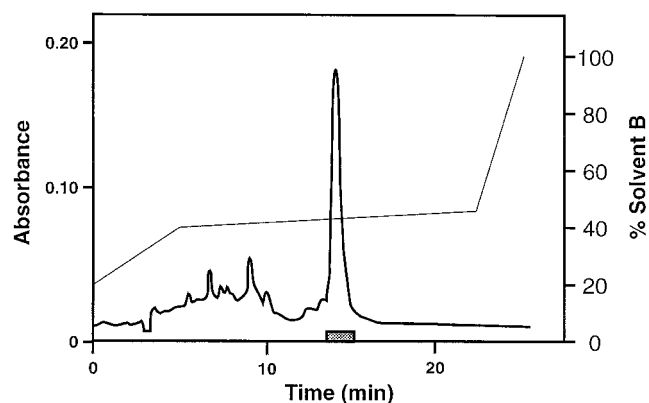


Figure 5. HPLC purification of UMP/CMP kinase. The GST-UMP/CMP kinase fusion protein was digested with enterokinase while still attached to the glutathione-Sepharose beads. The eluate was applied to a 250- × 10-mm, i.d., reverse-phase C_{18} column. A_{214} was monitored. The eluted protein peak containing UMP/CMP kinase activity was collected ($B = 41\%$). The hatched area under the peak illustrates the collected pool of UMP/CMP kinase.

was sequenced. The N-terminal sequence MGSVDAANGS-GKKPT was found to be identical to the amino acid sequence deduced from the cDNA. This sequence also indicates that the enterokinase cuts exactly at the predicted site, leaving no extra amino acids at the N terminus.

The amino acid composition of the expressed protein was also determined. About 1 μg of HPLC-purified UMP/CMP kinase was hydrolyzed in 6 N HCl for 65 min at 150°C (PICO-TAG Workstation, Waters). The free amino acids were derivatized under basic conditions with phenyl isothiocyanate in a derivatizer (model 420A, Applied Biosystems) and separated on a narrow-bore C_{18} column. The phenylthiocarbonyl chromophore was detected at A_{254} . Quantitation was performed from the A_{254} and comparison with a norleucine internal standard. The total amino acid composition was determined independently two times (data not shown). Gln and Asn could not be detected because both amino acids were converted to the corresponding carboxylic acids. Therefore, the resulting Asp and Glu were the sum of Asp and Asn (Asx) and of Glu and Gln (Glx), respectively. The number of Trp and Cys residues could not be determined by this method because they were destroyed during acid hydrolysis. There was good agreement between the independent analyses. A χ^2 analysis indicated with greater than 99% confidence that the composition found in the purified protein was the same as that of the predicted protein, confirming that the purified protein was the Arabidopsis UMP/CMP kinase.

Initial Rate Studies

Once we were convinced that the purified protein was indeed the Arabidopsis UMP/CMP kinase, the kinetic parameters of this purified protein were determined (Table I). The enzyme can utilize CMP as a phosphate donor almost as well as it does UMP. The K_m for UMP was 5- to 6-fold higher than that for ATP. Therefore, the enzyme binds ATP much more tightly than UMP. However, the K_m for CMP

was about equal to the K_m for ATP, indicating that the enzyme binds CMP as tightly as ATP. The k_{cat} obtained for each substrate was relatively the same.

pH Dependence of the Enzyme

The values of k_{cat} were determined at a series of different pH values from 5.5 to 8.0. All pH buffers contained both 50 mM Mes and 50 mM Hepes. When ATP was the variable substrate, the concentration of UMP was fixed at 400 μM . When UMP was the variable substrate, the concentration of ATP was fixed at 300 μM . The values of k_{cat} were obtained at pH 5.5, 6.5, 7.0, 7.5, and 8.0 when ATP or UMP was the variable substrate (data not shown). These analyses showed that optimal activity was achieved at pH 6.5 for both ATP and UMP.

Thermal Stability

The enzyme was heated for 10 min at various temperatures between 30 and 100°C. Residual activity was determined with 300 μM ATP and 400 μM UMP (data not shown). One-half of the enzymatic activity was maintained when the enzyme was heated at 58°C. Although still high, this is about 10°C lower than that of the *E. coli* enzyme.

Kinetic Mechanism

To better understand the kinetic mechanism, enzyme assays were conducted in which one substrate was varied at different fixed concentrations of other substrates. Double-reciprocal plots of these data showed that, when the ATP concentration was varied at different fixed levels of UMP, a family of lines intersecting in the second quadrant was obtained (Fig. 7A). A similar family of lines was obtained for various 1/UMP concentrations at different fixed levels of ATP (Fig. 7B). These families of lines are theoretical, obtained from Equation 1 when $n = 1$. The data obtained from the experimental evaluation matches the theoretical family of lines for both ATP and UMP. Based on the closeness of this fit, we conclude that the enzyme fits a

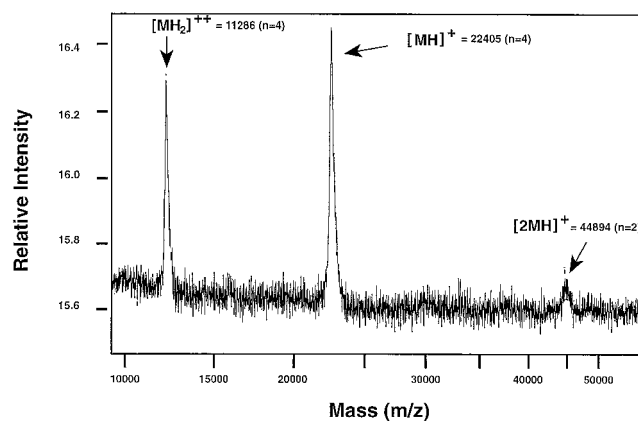


Figure 6. Typical mass spectrum of *A. thaliana* UMP/CMP kinase. The $[\text{MH}_2]^{2+}$ peak is at 11,286, the $[\text{MH}]^+$ peak is at 22,405, and the $[2\text{MH}]^+$ peak is at 44,984.

Table 1. Kinetic parameters of *A. thaliana* UMP/CMP kinase

The standard enzyme reaction contained 50 mM Mes, pH 6.5, 50 mM KCl, 2 mM MgCl₂, 1 mM PEP, 0.2 mM NADH, 3.5 units of pyruvate kinase, and 5 units of lactate dehydrogenase in a final volume of 1 mL. When ATP was used as the variable substrate, UMP and CMP were fixed at 400 and 450 μM, respectively. When UMP or CMP were used as the variable substrate, ATP-Mg²⁺ was fixed at 300 μM for UMP and at 800 μM for CMP. The reaction was started by the addition of UMP/CMP kinase. The change in A₃₄₀ was recorded. One unit of UMP kinase is defined as the amount of enzyme that catalyzed the formation of 1 μmol of UDP or CDP per min.

| Activity | Specific Activity <i>units mg⁻¹ × 10⁻²</i> | <i>K_m</i> | | | <i>k_{cat}</i> <i>s⁻¹</i> |
|------------|---|----------------------|------------------|--------------|---|
| | | ATP | UMP <i>μM</i> | CMP | |
| UMP kinase | 3.67 ± 0.12 | 29.3 ± 2.8 | 152.9 ± 14.5 | – | 7.64 ± 0.24 |
| CMP kinase | 4.12 ± 0.05 | 291.7 ± 24.3 | – | 266.4 ± 21.7 | 8.57 ± 0.11 |

random Bi-Bi mechanism. The data fit the random Bi-Bi mechanism shown in Equation 1 (Fromm, 1975):

$$\frac{1}{V} = \frac{1}{V_m} \left[1 + \frac{K_a}{A^n} + \frac{K_b}{B} + \frac{K_{ia}K_b}{A^n B} \right] \quad (1)$$

where *V*, *V_m*, *A*, *B*, *K_a*, *K_b*, and *K_{ia}* represent the initial velocity, maximum velocity, concentration of free ATP, concentration of free UMP, Michaelis constant for ATP, Michaelis constant for UMP, and dissociation constant for ATP, respectively. The Hill coefficient for ATP is represented by *n*. *K_{ib}* is the dissociation constant for UMP and substitutes into Equation 1 in place of *K_{ia}*. *K_{ia}* and *K_{ib}* were determined experimentally. *K_{ia}* (for ATP) = 14.9 ± 1.3 μM and *K_{ib}* (for UMP) = 110.2 ± 8.7 μM.

Alternative Substrates

To examine the specificity of the UMP/CMP kinase in more detail, various phosphate donors and acceptors were evaluated for their ability to function in this enzyme assay. To evaluate the phosphate acceptors, 300 μM ATP was used as the donor with different monophosphate acceptors at 400 μM. Only UMP and CMP are effective phosphate acceptors. Neither orotidine monophosphate nor TMP were effective as a phosphate acceptor. The presence of the 2' hydroxyl on the Rib moiety is also important because dUMP and dCMP are 30-fold less active than their ribosyl analogs (data not shown). None of the purine monophosphates tested (AMP, GMP, inosine monophosphate, and xanthine monophosphate) functioned effectively as a phosphate acceptor. Similarly, other nucleotide triphosphates were examined for their ability to function as phosphate donors to UMP. For evaluation, 300 μM phosphate donors were used with UMP at 400 μM. In this analysis, only ATP and dATP were effective phosphate donors, with dATP only half as effective as ATP. Other purine triphosphates (GTP, dGTP, inosine triphosphate, and xanthine triphosphate) were 20- to 30-fold less active than ATP. Pyrimidine triphosphates (UTP, CTP, dCTP, and deoxyribothymine triphosphate) were 70-fold less active than ATP.

It is known that the *E. coli* UMP kinase is allosterically regulated by both GTP and UTP (Serina et al., 1995). With the *E. coli* enzyme, 100 μM GTP activates the enzyme 5-fold and 100 μM UTP down-regulates the enzyme 5-fold. We therefore explored whether these nucleotides affected the activity of the Arabidopsis enzyme. Neither of these nucle-

otides significantly affected the activity of the Arabidopsis UMP/CMP kinase, even at extremely high levels. At 3 mM GTP enzyme activity increased by 25%, but this is much lower than the 5-fold activation of the *E. coli* enzyme that occurs with lower levels of GTP (Serina et al., 1995). Conversely, UTP also affects the UMP/CMP kinase activity but, again, only by 25% at UTP concentrations as high as 3 mM. Therefore, whereas GTP and UTP both affect the plant enzyme, the effect of each of these nucleotides even at very high levels is significantly less than the effect on the prokaryotic enzyme.

Kinetics of Ap₅A Inhibition

We also examined the inhibition of the Arabidopsis UMP/CMP kinase with the bifunctional inhibitor Ap₅A (Fig. 8). The inhibitor concentration required for 50% inhibition was determined by fixing ATP at 300 μM and UMP at 400 μM and varying the Ap₅A concentration between 0 and 100 μM. The inhibitor concentration for 50% displacement of Ap₅A on the Arabidopsis enzyme was 14 μM, which is lower than that found on the enzyme from either *D. discoideum* (Weismüller et al., 1990) or *E. coli* (Serina et al., 1995). The inhibition mechanism of Ap₅A was determined by fixing one substrate at a saturating concentration and varying the concentration of the other substrate at different fixed concentrations of Ap₅A. The families of lines shown in Figure 8 are theoretically fit to Equation 2 (Fromm, 1975) and the points were experimentally derived.

$$\frac{1}{V} = \frac{1}{V_m} \left[1 + \frac{K_a}{A} \left(1 + \frac{I}{K_i} \right) \right] \quad (2)$$

where *I*, *K_a*, and *K_i* represent the concentration of free Ap₅A, the Michaelis constant for ATP (or UMP), and the inhibition constant for Ap₅A, respectively. The inhibition by Ap₅A when ATP was the variable substrate is shown in Figure 8A, and the inhibition of Ap₅A when UMP was the variable substrate is shown in Figure 8B. These data clearly demonstrate that Ap₅A is a competitive inhibitor of both ATP and UMP. The *K_i* values for ATP and UMP were 1.20 ± 0.02 and 6.53 ± 0.03 μM, respectively.

DISCUSSION

We have isolated the cDNA encoding the *A. thaliana* UMP/CMP kinase by complementation of an *S. cerevisiae*

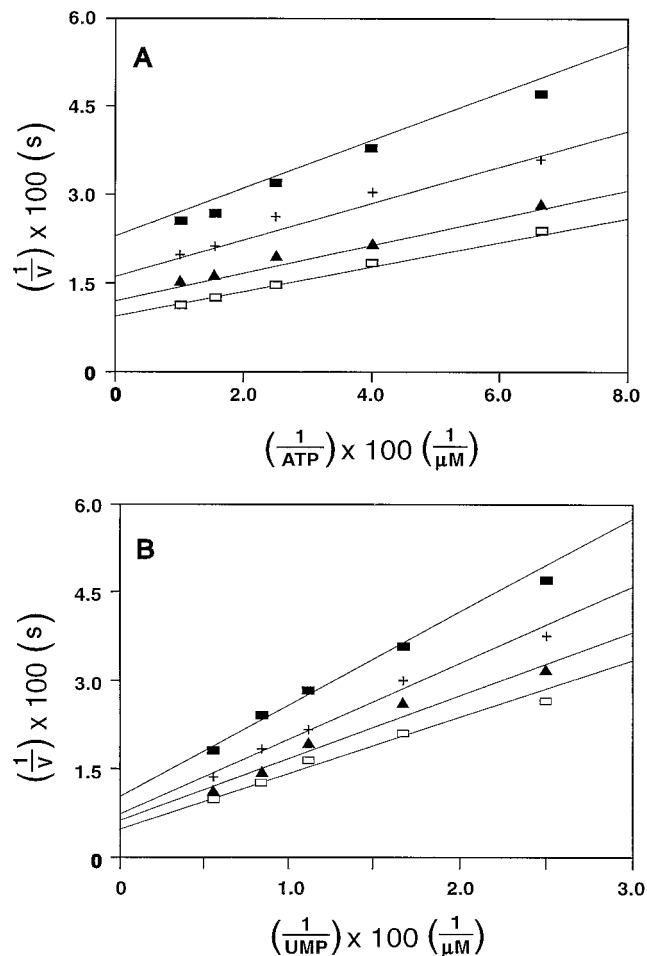


Figure 7. Analysis of kinetic mechanism. A, Double-reciprocal plot of initial velocity versus ATP concentrations. For these experiments the concentrations of UMP were 40 (■), 60 (+), 90 (▲), and 120 μM (□). The lines are theoretical based on Equation 1 where $n = 1$. The points were experimentally determined. B, Double-reciprocal plot of initial velocity versus UMP concentrations. For these experiments, the concentrations of ATP were 15 (■), 25 (+), 40 (▲), and 65 μM (□). The lines are theoretical based on Equation 1 where $n = 1$. The points were experimentally determined.

UMP kinase mutant. Complementation of known yeast mutants is a very effective way to obtain eukaryotic genes. Following isolation, the plant cDNA was characterized by sequencing. The full-length cDNA encodes a 202-amino acid protein that is closely related to UMP kinases from other eukaryotic sources. The plant enzyme, however, did not share high identity with the bacterial or archaeobacterial UMP kinases. In previous studies, the *E. coli* UMP kinase was found to belong to the aspartokinase family (Serina et al., 1995).

When the intracellular location of the Arabidopsis UMP kinase was examined using the online analysis tool PSORT (Nakai and Kanehisa, 1992), a cytosolic localization for the enzyme was predicted. A cytosolic localization has also been found for the rice adenylate kinase enzyme (Kawai and Uchimiya, 1995). The cytosolic localization is also con-

sistent with the finding that uridine nucleotides are predominantly located in the cytosol (Dancer et al., 1990).

After characterization of the cDNA, the coding region was expressed by fusing it to GST. Following expression, the fusion protein was cleaved and the Arabidopsis UMP/CMP kinase was purified by HPLC. The molecular mass, amino acid composition, and N-terminal sequence of the expressed protein all demonstrated that it was indeed UMP/CMP kinase. When examined for enzyme activity, the purified enzyme was found to have both UMP kinase and CMP kinase activity. Kinetic parameters were deter-

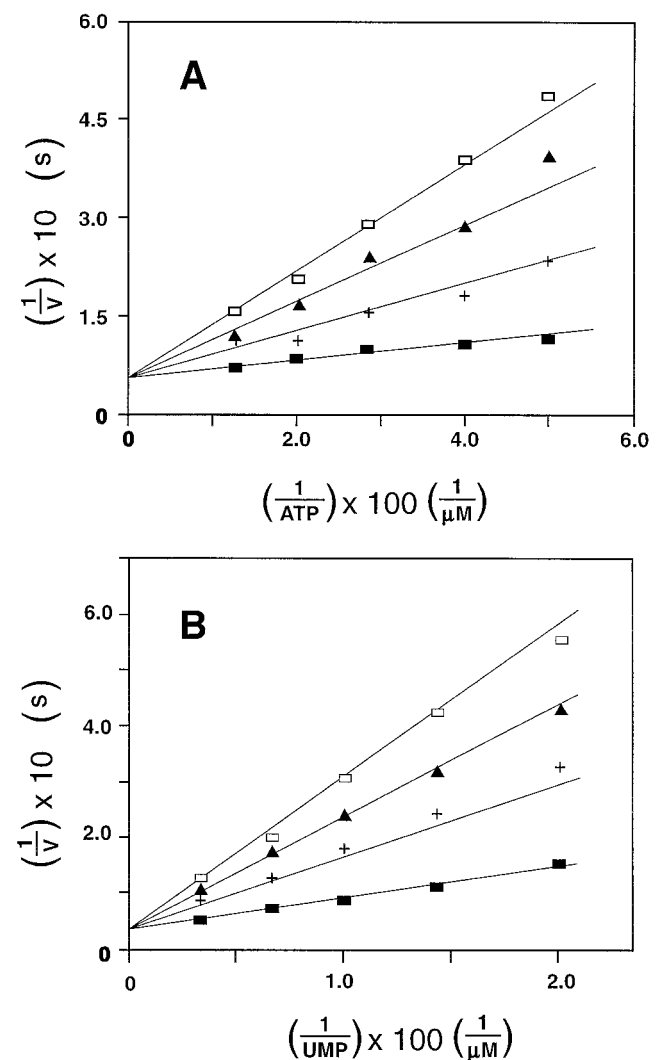


Figure 8. Kinetics of Ap₅A inhibition. A, Double-reciprocal plot of initial velocity versus ATP concentrations in the presence of Ap₅A inhibitor. UMP concentrations were fixed at 400 μM . The concentrations of Ap₅A were 0 (■), 2 (+), 4 (▲), and 6 μM (□). The lines are theoretical based on Equation 2. The points were experimentally determined. B, Plot of reciprocal of initial velocity of UMP/CMP kinase versus reciprocal of UMP concentrations in the presence of Ap₅A inhibitor. ATP concentrations were fixed at 300 μM . The concentrations of Ap₅A were 0 (■), 8 (+), 16 (▲), and 24 μM (□). The lines are theoretical based on Equation 2. The points were experimentally determined.

mined for the plant enzyme and several differences from the *E. coli* enzyme were noted.

First, the *E. coli* enzyme shows remarkable thermal stability in the absence of protective agents, being stable up to 65°C. Although relatively thermostable, the Arabidopsis enzyme did not show the same degree of stability. Second, the plant enzyme is not allosterically regulated in the same way that the prokaryotic enzyme is regulated. At concentrations that dramatically affect the enzyme activity of the *E. coli* UMP kinase, neither GTP nor UTP have a significant effect on the plant enzyme. Indeed, at 30 times the level that affects the bacterial enzyme, the plant enzyme is affected by only about 25%. Bourne et al. (1991) have identified a pair of sequences, Asp-77-His-Met-Gly-80 and Thr-165-Lys-Val-Asp-168, that are conserved in GTP-binding proteins. Both of these sequences are conserved in the *E. coli* UMP kinase (Serina et al., 1995). However, neither of them is present in the Arabidopsis UMP/CMP kinase. Thus, by both sequence identity and experimental observation, allosteric regulatory sites are not present in the plant enzyme, and the Arabidopsis enzyme seems to be both functionally and structurally different from the prokaryotic enzyme.

The K_m values for UMP and CMP indicate that the Arabidopsis enzyme can utilize both pyrimidine monophosphates equally well as phosphate acceptors. This is similar to the enzyme from rat Novikoff ascites tumors (Orengo and Maness, 1978), but the mouse enzyme utilizes UMP nearly twice as effectively as CMP as a phosphate acceptor (Andersen, 1978a). The deoxy forms are almost totally ineffective as phosphate acceptors in the Arabidopsis enzyme. Two important conclusions can be drawn from this finding. First, a different enzyme must be responsible for the conversion of dCMP into dCDP. This is different from the mouse and the *Tetrahymena pyriformis* enzyme, in which dCMP can also act as a phosphate acceptor (Andersen, 1978b). Second, because the nucleotide monophosphate-binding pocket does not discriminate between UMP and CMP, the exclusion of deoxynucleotides results not only in the exclusion of dCMP but also in the exclusion of dUMP. Thus, the conversion of dUMP into dUDP does not occur, thereby forcing the conversion of dUMP into TMP by thymidylate synthase. Furthermore, TMP is ineffective as a phosphate acceptor, indicating that, as in yeast (Jong et al., 1984), a separate TMP kinase must also exist.

Structural studies of adenylate kinase from both *E. coli* (Müller and Schulz, 1992) and beef heart (Diederichs and Schulz, 1991) revealed that the 2' hydroxyl of the phosphate acceptor forms a strong hydrogen bond with an α -chain carboxyl. It is likely that similar interactions are required in the plant UMP/CMP kinase, thereby biasing the specificity against the deoxynucleotides.

Eukaryotic UMP kinases in general have a higher specificity for ATP as the phosphate donor, with dATP effective at about 10% the level of ATP (Orengo and Maness, 1978). This was also found for the plant enzyme. Other nucleotide triphosphates were essentially ineffective as phosphate donors.

The plant UMP kinase has a high degree of identity with other eukaryotic UMP kinases and are expected to share significant structural identity. The UMP kinase enzyme from yeast has been purified and characterized (Ma et al., 1990). The crystal structure of this enzyme has been solved with substrates in place (Müller-Dieckmann and Schulz, 1994, 1995). The substrates are held in position by numerous favorable contacts with the protein. Most of these contacting residues are conserved between the yeast enzyme and the plant enzyme.

Structural studies have also demonstrated that the UMP-binding pocket of the yeast enzyme is of sufficient size to accommodate an AMP moiety (Müller-Dieckmann and Schulz, 1994, 1995). This explains the high activity of the yeast UMP kinase for AMP. The finding that $A_{p_5}A$ is a competitive inhibitor of UMP with a micromolar K_i indicates that the Arabidopsis enzyme has a UMP-binding pocket that is also sufficiently large to accommodate an AMP moiety. However, the Arabidopsis enzyme has less than 0.5% activity with AMP. Therefore, the structure of the UMP-binding pocket of the Arabidopsis enzyme will be particularly interesting to understand. The radiography-crystallographic studies of the yeast UMP kinase have failed to explain the specificity of this enzyme for UMP. Those residues that have been shown to line the uracil-binding pocket of the yeast UMP kinase (Ala-47, Leu-51, Ile-75, Val-76, Thr-81, Phe-105, Arg-107, and Gln-111) are, with only one exception (Asn substitutes for Gln), completely conserved in the Arabidopsis enzyme. Note that this same substitution (Asn for Gln) is found in the *D. discoideum* enzyme, which also shows a high degree of substrate discrimination for UMP over AMP (Weismüller et al., 1990).

The enzymatic mechanism is also relatively well understood for the yeast enzyme. The transition state of phosphoryl transfer is maintained by a scaffold of interactions, including the C $^{\alpha}$ -backbones of the CORE domains and a series of six positively charged residues (Lys-29, Arg-52, Arg-107, Arg-142, Arg-148, and Arg-159) positioned to coordinate the phosphates. All of these residues are conserved in the Arabidopsis enzyme. The interaction of the substrate-fixed phosphates with the yeast UMP kinase is virtually identical to *E. coli* adenylate kinase (Müller-Dieckmann and Schultz, 1994), indicating the widespread and general conservation of this enzymatic mechanism. Because of the high degree of conservation in these important contacting residues, it is probable that the Arabidopsis enzyme also shares this enzymatic mechanism.

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