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# Site-Specific Mutations of Conserved Residues in the Phosphate-Binding Loop of the *Arabidopsis* UMP/CMP Kinase Alter ATP and UMP Binding[1]

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**All eukaryotic UMP/CMP kinases contain a glycine-rich sequence GGPG(S/A)GK at the N-terminus. This sequence is homologous to the conserved sequence GXXGXGK found in other ATP-binding proteins. To study the role of this conserved sequence in *Arabidopsis* UMP/CMP kinase, five conserved residues were mutated by site-directed mutagenesis to generate seven mutant enzymes: G21A, G22A, G24A, G26A, K27R, K27M, and K27E. The G21A and G26A mutants were degraded during the purification phase and were thus unable to be purified. Kinetic studies on the other mutants, when compared to studies on the wild-type enzyme, revealed that this sequence is important for ATP binding and enzyme catalysis. All mutants had a decreased $k_{cat}/K_m^{ATP}$ value. The G22A and G24A mutants had about half of the $k_{cat}$ value of wildtype and 3.9-fold and 3.3-fold increases in $K_m^{ATP}$ values, respectively. The $k_{cat}/K_m^{ATP}$ values in the K27M and K27E mutants were changed significantly and decreased by 1000-fold and 2600-fold, respectively. The removal of the terminal positive charge of Lys27 in the K27M and K27E mutants resulted in 20% of the $k_{cat}$ value of wildtype. However, both mutants had a remarkable increase in $K_m^{ATP}$ value by 241-fold and 552-fold, respectively. Therefore, the positive charge of Lys27 plays an important role on both ATP binding and enzyme catalysis. Interestingly, the results also showed that the mutations that affected ATP binding also had an effect on UMP binding.** © 1998 Academic Press

***Key Words:*** **UMP kinase; CMP kinase; site-specific mutagenesis; *Arabidopsis thaliana;* ATP binding; phosphate-binding loop.**

Adenine nucleotide-binding proteins are involved in many biological processes, such as membrane transport, cell division, protein export, and DNA repair (1, 2). These proteins share a unique structural feature among them: a glycine-rich phosphate-binding loop, GXXGXGK (X represents an unspecified amino acid residue), is found in the amino acid sequence near the N-terminus of the protein (2, 3). Similar N-terminal glycine-rich sequences are found in other nucleotide-binding proteins, including other NMP kinases (3, 4), GTP binding proteins (5, 6), and enzymes such as adenylosuccinate synthetase (7). The glycine-rich, conserved phosphate-binding loop is important for nucleotide binding and forms a large anion hole that accommodates phosphates of ATP (8).

UMP/CMP kinase catalyzes the transfer of phosphoryl group from ATP to either UMP or CMP to form ADP and UDP or CDP. All pyrimidines within the cell are derived from UMP. Therefore, UMP kinase catalyzes the first committed step of pyrimidine metabolism. The genes or cDNAs encoding UMP kinase have been cloned from *Escherichia coli* (9), *Dictyostelium discoideum* (10), *Saccharomyces cerevisiae* (11), *Sus scrofa* (12), and *Arabidopsis thaliana* (13). The *Arabidopsis* enzyme shares 47 to 53% identity with the UMP kinase enzymes prepared from these respective enzymes. However, the *Arabidopsis* UMP/CMP kinase has only a limited amount of identity with AMP kinases, sharing 19% identity with the AMP kinase from *S. cerevisiae* (14). Alignment of the amino acid sequences of all four eukaryotic UMP kinases reveals that a glycine-rich

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sequence, GGPG(S/A)GK, found in the N-terminal region, is conserved in all the enzymes.

The crystal structures of UMP kinases from *S. cerevisiae* and *D. discoideum* have been described (15–17). The crystal structure of UMP/CMP kinase from *D. discoideum* complexed with a bisubstrate inhibitor $UP_5A$[3] with a resolution of 2.2 Å showed that the globular enzyme consisted of eight $\alpha$-helixes that surround five parallel $\beta$-sheets. The phosphate-binding loop was located between a $\beta$-sheet ($\beta$1) and an $\alpha$-helix ($\alpha$2). The residues of this loop (Gly13, Gly14, Gly16, and Lys19) along with several conserved arginines formed interactions with the five phosphoryl groups of $UP_5A$ in the active center of the enzyme. The structure also showed that the $\epsilon$-amino group of Lys19 in the phosphate-binding loop interacted strongly with oxygens of P2 (corresponding to $\beta$-phosphoryl group of ATP) and P4 of $UP_5A$ and is stabilized additionally by the main-chain carbonyl group of Gly14. The Lys19 together with $Mg^{2+}$ is responsible for the positioning of the reacting phosphoryl groups. A similar situation was found for the phosphate-binding loop in the yeast enzyme. Therefore, the residues in the phosphate-binding loop, especially Lys19, are important in ATP binding and/or enzyme catalysis. Any change in the conserved residues may result in alterations in the folding pattern and nucleotide binding (18–20).

In order to study the structure and function relationship of *Arabidopsis* UMP/CMP kinase, we chose to study the phosphate-binding sequence of ATP by site-directed mutagenesis. We made several mutations in the phosphate-binding loop of the *Arabidopsis* UMP/CMP kinase. The kinetic studies showed that this phosphate-binding loop is important for ATP binding and enzyme activity as predicted from the available crystal structures from other sources.

## EXPERIMENTAL PROCEDURES

*Materials.* The pGEX-4T-3 and glutathione Sepharose 4B were from Pharmacia Biotech (Piscataway, NJ). The vector pBluescript II SK (+/−) and *Pfu* DNA polymerase were from Stratagene (La Jolla, CA). Enterokinase was from Biozyme Laboratories (San Diego, CA). Restriction enzymes and T4 DNA Ligase were from Promega (Madison, WI). Oligonucleotides were synthesized at the Nucleic Acid Facility of Iowa State University. All other enzymes and reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. The bacterial strain XL1-Blue was used for all bacterial manipulations and expression.

*Site-directed mutagenesis by PCR and construction of mutant plasmids.* Single-site mutations of amino acid residues were introduced by two rounds of PCR (21). Figure 1 summarizes the strategy used to generate the mutation. All primers used are listed in Fig. 1B. The design of primer P1 was based on the sequence of the vector pGEX-4T-3. Primers P2 and P3 contained a specific mutation for one mutant. Primer P4 was based on the sequence of the vector pRT380. This primer also contained a *Xho*I site and an additional stop codon. The plasmid pRT380 was used as a template for all mutations in the first-round PCR. The plasmid pRT380 was constructed by inserting the 637-bp *Bam*HI/*Xho*I fragment containing the intact *Arabidopsis* UMP/CMP kinase open reading frame, an enterokinase site for cleavage of the GST fusion protein, and an additional stop codon into the *Bam*HI/*Xho*I sites of pGEX-4T-3. The first-round PCR was performed in two separate reactions using two sets of primers P1 and P3 or P2 and P4. Depending on which PCR oligonucleotides were used for the different mutants, PCR products of about 320 and 560 bp were gel purified and used as templates in the second-round PCR using primer P1 and P4. The 880-bp PCR product was digested with *Bam*HI and *Xho*I. The resulting 637-bp fragment was subcloned into pBluescript II SK (+/−), and the mutation was confirmed by sequencing using T3 and T7 primers. The 637-bp *Bam*HI/*Xho*I fragment then was excised from pBluescript II SK (+/−) and subcloned into pGEX-4T-3. Each mutant cDNA construct was transformed to bacterial strain XL1-Blue and confirmed by sequencing using primers on the vector. The clones that had been verified by sequencing were further used for the expression and purification of the corresponding mutants.

*Purification and kinetic studies of wild-type and mutant UMP/CMP kinases.* The protocols for expression and purification of the mutant UMP/CMP kinases were the same as those for the wild-type enzyme (13). Protein purity was analyzed by SDS–PAGE according to the method of Laemmli (22). Protein concentration was determined by Bradford assay with bovine serum albumin as a standard (23). UMP kinase activity was determined as described previously (13). Substrate saturation levels were determined independently for each mutant protein and these values are presented in Table I.

$K_m$ values for each substrate were obtained by keeping the other substrate at the saturating level (3–10 times its $K_m$ value) and varying the concentration of the test substrate. Substrate inhibition was not found with any of the enzymes tested. All kinetic data were analyzed using the computer program ENZFITTER (24). The $V_{max}$ value used for calculating the $k_{cat}$ value was the average of five initial rates at saturating concentrations of both ATP and UMP substrates. To double check the $K_m$ and $V_{max}$ values, double-reciprocal plots were also constructed. The data from ENZFITTER agreed well with the data from the double-reciprocal plots.

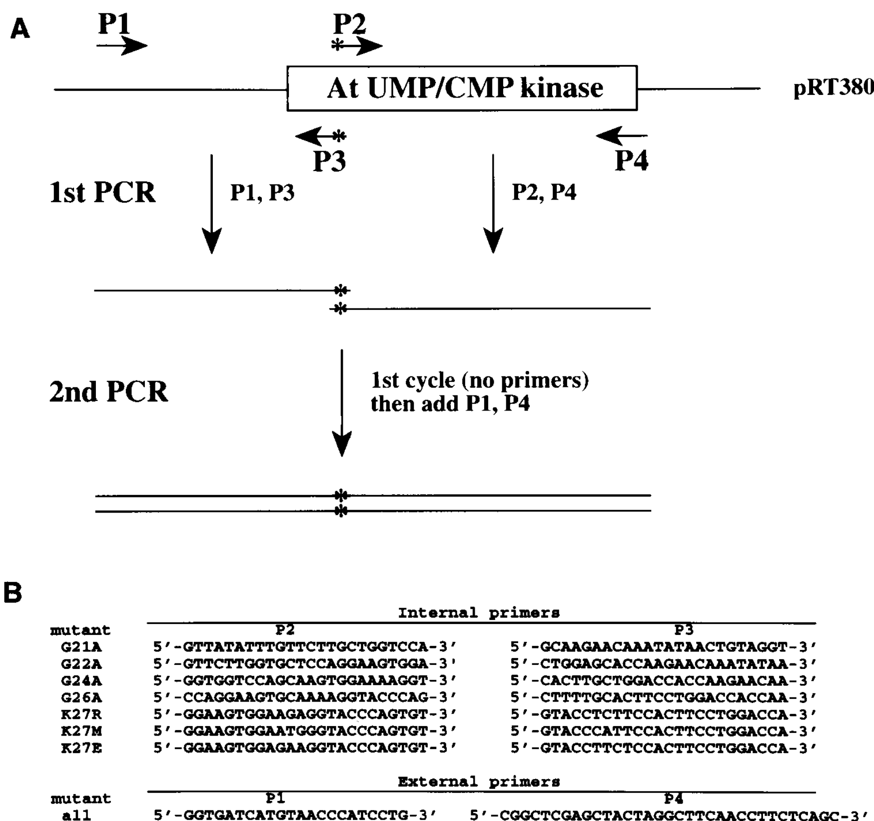
*Matrix-assisted laser desorption ionization (MALDI) mass spectroscopy.* Protein samples of 0.5 to 1.0 $\mu$l containing about 0.5 to 1 $\mu$g of protein were loaded with 0.5 $\mu$l of freshly prepared 3,5-dimethoxy-4-hydroxy cinnamic acid matrix into a Finnigan LASERMAT 2000 MALDI-time of flight mass analyzer. The collected data were analyzed using the LASERMAT 2000 data processing software. Lysozyme was used as an internal calibration standard. The molecular mass found for each protein was in good agreement with the expected molecular masses of the protein (data not shown).

*Circular dichroism spectroscopy (CD).* CD studies on the wild-type and mutant forms of UMP/CMP kinase were performed in 20 mM MES buffer (pH 6.5) at room temperature in a JASCO CD Model J-710 spectrometer. Samples were placed in a 1-mm cuvette, and data points were collected from 190 to 260 nm in 0.5-nm increments. Each spectrum was calibrated to remove the background of the buffer and smoothed by using the Standard Analysis program provided with the spectrometer.

## RESULTS

*Mutagenesis of UMP/CMP kinase cDNA.* The alignment of amino acid sequences of the eukaryotic UMP/CMP kinases demonstrated the presence of the ATP-binding consensus sequence, GXXGXGK, which is homologous to the conserved sequence GXXGXGK found in many other nucleotide-binding proteins (2–7). This

[3] Abbreviation used: $UP_5A$, $P^1$-(5′-adenosyl) $P^5$-(5′-uridyl) pentaphosphate.




| mutant | Internal primers P2 | Internal primers P3 |
|---|---|---|
| G21A | 5′-GTTATATTTGTTCTTGCTGGTCCA-3′ | 5′-GCAAGAACAAATATAACTGTAGGT-3′ |
| G22A | 5′-GTTCTTGGTGCTCCAGGAAGTGGA-3′ | 5′-CTGGAGCACCAAGAACAAATATAA-3′ |
| G24A | 5′-GGTGGTCCAGCAAGTGGAAAAGGT-3′ | 5′-CACTTGCTGGACCACCAAGAACAA-3′ |
| G26A | 5′-CCAGGAAGTGCAAAAGGTACCCAG-3′ | 5′-CTTTTGCACTTCCTGGACCACCAA-3′ |
| K27R | 5′-GGAAGTGGAAGAGGTACCCAGTGT-3′ | 5′-GTACCTCTTCCACTTCCTGGACCA-3′ |
| K27M | 5′-GGAAGTGGAATGGGTACCCAGTGT-3′ | 5′-GTACCCATTCCACTTCCTGGACCA-3′ |
| K27E | 5′-GGAAGTGGAGAAGGTACCCAGTGT-3′ | 5′-GTACCTTCTCCACTTCCTGGACCA-3′ |

| mutant | External primers P1 | External primers P4 |
|---|---|---|
| all | 5′-GGTGATCATGTAACCCATCCTG-3′ | 5′-CGGCTCGAGCTACTAGGCTTCAACCTTCTCAGC-3′ |

**FIG. 1.** PCR method for generation of mutants. (A) The P1, P2, P3, and P4 represent the four primers used in PCR methodology. The vector, pRT380, is the expression construct containing the *Arabidopsis* wild-type UMP/CMP kinase cDNA. The specific mutation site is shown as an asterisk (*). (B) The specific primers used to generate each mutant.

suggested that this glycine-rich sequence in *Arabidopsis* UMP/CMP kinase may play as important a role in ATP binding as it does in other nucleotide-binding proteins. Therefore, site-directed mutagenesis was conducted to introduce a series of the mutations within this consensus sequence of *Arabidopsis* UMP/CMP kinase. The conserved glycine residues at positions 21, 24, and 26 were changed to alanine. The glycine residue at position 22 was conserved among all four eukaryotic UMP kinases. Therefore, it was also mutated to alanine. The conserved lysine at position 27 was changed to arginine, methionine, or glutamic acid. The arginine mutant (K27R) was prepared to retain the terminal positive charge. The methionine mutant (K27M) was designed to remove the terminal positive charge of lysine while maintaining the long alkyl side chain. The glutamic acid mutant (K27E) was used to remove the terminal positive charge and introduce a negative charge. These three mutants at the same position were expected to interpret any differences in kinetic data caused by change of size or charge of the lysine residue.

**TABLE I**

Saturating Concentrations for UMP and ATP for Each Mutant UMP/CMP Kinase[a]

| Mutant | Saturated [UMP] | Saturated [ATP] |
|---|---|---|
| Wildtype | 400$\mu$M | 300 $\mu$M |
| G22A | 1000$\mu$M | 900 $\mu$M |
| G24A | 900$\mu$M | 800 $\mu$M |
| K27R | 700$\mu$M | 500 $\mu$M |
| K27M | 1000$\mu$M | 45mM |
| K27E | 400$\mu$M | 9mM |

[a] For each mutant the kinetic values were determined at saturating concentration of ATP and UMP as follows. First, the concentration of UMP was set to 400 mM, which is the UMP saturated concentration for wildtype UMP/CMP kinase. Next we varied the ATP concentration to obtain the ATP concentration giving the maximal velocity. Then using this ATP concentration, we varied the UMP concentration to obtain the UMP concentration giving the maximal velocity. This concentration was taken as the UMP saturated concentration for that particular mutant. Finally, holding the UMP concentration at saturation, ATP concentration was varied to get the saturation level of ATP for that particular mutant.

*Purification of the mutant enzymes.* The procedures used to purify the mutant enzymes were basically the same as that for the wild-type enzyme (13). Five of the mutant enzymes were successfully purified. The purity was detected on SDS–PAGE. Both the mutant and wild-type enzymes showed single bands (about 22 kDa)

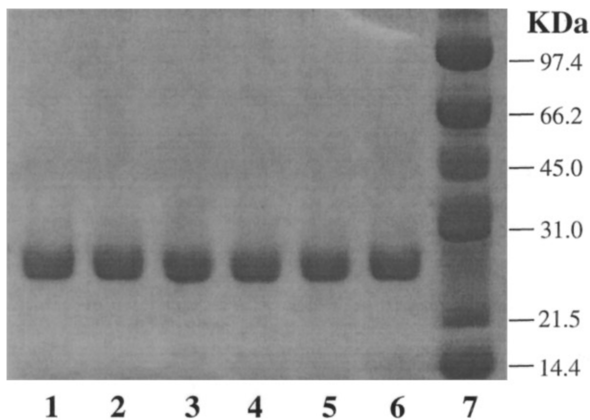



**FIG. 2.** SDS–PAGE analysis of purified wild-type and mutant UMP/CMP kinases. All samples were run on a 12% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lane 1, wild-type; lane 2, G22A; lane 3, G24A; lane 4, K27R; lane 5, K27M; lane 6, K27E; lane 7, molecular weight markers.

and had the same migration distance on the gel (Fig. 2). MALDI analysis verified that the molecular mass of all the mutant enzymes was the same as that of the wild-type enzyme (data not shown). However, the G21A and G26A mutants could not be purified. In contrast to the other mutant proteins, both of these mutant fusion proteins were unstable and were degraded during the purification process.

*Secondary structure analysis.* The secondary structures of the wild-type and mutant enzymes were analyzed by CD spectrometry. This CD study was used to check whether localized or global structural alterations were introduced by the various mutations. The CD spectral results indicated that the spectrum of each mutant was essentially superimposable on that of the wild-type enzyme (data not shown). Therefore, these CD results suggested that no major conformational changes occurred in any of the purified mutants compared to the wild-type enzyme.

*Kinetic analysis of UMP/CMP kinase mutants.* To determine the effect of the introduced mutations on substrate binding and enzyme activity, initial rate kinetic studies of the mutant UMP/CMP kinases were performed and the data were compared with those of the wild-type enzyme. These results, summarized in Table II, showed that each of the mutations caused a decrease in the $k_{cat}$ value compared to the wild-type enzyme. The $k_{cat}$ values for the G22A and G24A mutants were 4.5 and 3.7 $s^{-1}$, respectively, which are equivalent to about 50% of the wild-type activity 7.6 $s^{-1}$. Therefore, the mutations at positions 22 and 24 have a modest effect on enzyme catalysis. The greatest changes in the $k_{cat}$ values were observed in the mutations at position 27. The $k_{cat}$ values for K27R, K27M, and K27E were about 42, 22, and 21% of the $k_{cat}$ value of the wild-type activity, respectively.

In contrast to the $k_{cat}$ values, the $K_m^{ATP}$ values for all mutants except the K27R mutant increased. The $K_m^{ATP}$ values for the G22A and G24A mutants were 3.9-fold and 3.3-fold greater than that of the wild-type enzyme. The $K_m^{ATP}$ values for the K27M and K27E mutants showed the greatest changes. These $K_m^{ATP}$ values increased by 241-fold and 552-fold, respectively. On the other hand, the K27R mutant showed only a small decrease in the $K_m^{ATP}$ value.

Interestingly, all the mutations except the arginine substitution at position 27 also had an effect on the $K_m^{UMP}$ value for UMP. The $K_m^{UMP}$ values for the G22A and G24A mutants increased by 2.5-fold and 13.4-fold, respectively. However, the $K_m^{UMP}$ values for the K27M and K27E mutants decreased by 8.5-fold and 25.5-fold, respectively. The $K_m^{UMP}$ value for the K27R mutant showed almost no change.

## DISCUSSION

*Arabidopsis* UMP/CMP kinase, like other eukaryotic UMP kinases and many other nucleotide-binding proteins, contains a conserved glycine-rich sequence near

**TABLE II**

Kinetic Parameters of Wild-Type and Mutant UMP/CMP Kinases from *Arabidopsis thaliana*[a]

| Protein | $k_{cat}$ ($s^{-1}$) | $K_m^{ATP}$ ($\mu$M) | $K_m^{UMP}$ ($\mu$M) | $k_{cat}/K_m^{ATP}$ $10^{-2}$ (s $\mu$M)$^{-1}$ | $k_{cat}/K_m^{UMP}$ $10^{-2}$ (s $\mu$M)$^{-1}$ |
|---|---|---|---|---|---|
| Wild-type | 7.6 ± 0.2 | 29 ± 3 | 153 ± 15 | 26.0 ± 0.3 | 5.0 ± 0.0 |
| G22A | 4.5 ± 0.1 | 115 ± 8 | 382 ± 34 | 3.9 ± 0.0 | 1.2 ± 0.0 |
| G24A | 3.7 ± 0.0 | 96 ± 9 | 2050 ± 50 | 3.9 ± 0.0 | 0.1 ± 0.0 |
| K27R | 3.4 ± 0.3 | 22 ± 4 | 133 ± 7 | 15.4 ± 0.6 | 2.6 ± 0.0 |
| K27M | 1.7 ± 0.1 | 7 ± 1 × $10^3$ | 18 ± 2 | (2.6 ± 0.1)$10^{-2}$ | 9.8 ± 0.1 |
| K27E | 1.6 ± 0.0 | 16 ± 1 × $10^3$ | 6 ± 1 | (1.0 ± 0.0)$10^{-2}$ | 27.1 ± 1.2 |

[a] The standard enzyme reaction contained 50 mM MES, pH 6.5, 50 mM KCl, 2 mM $MgCl_2$, 1 mM phosphoenoylpyruvate, 0.2 mM NADH, nucleotides, 3.5 units of pyruvate kinase, and 5 units of lactate dehydrogenase in a final volume of 1 ml. When one substrate was used as the variable substrate, the other substrate was fixed at the saturating concentration. The reaction was started by addition of UMP/CMP kinase. The change in optical density at 340 nm was recorded. One unit of UMP/CMP kinase is defined as the amount of enzyme that catalyzed the formation of 1 $\mu$mole of UDP or CDP per min.

the N-terminus of the polypeptide chain. The crystal structures of UMP kinases from yeast and *D. discoideum* suggested that this consensus sequence may play a role in ATP binding and/or enzyme catalysis (15–17). The site-directed mutagenesis studies in this report support this suggestion. Substitutions of glycine residues with alanine at positions 22 and 24 and lysine at position 27 with arginine, methionine, or glutamic acid all resulted in a decrease in the $k_{cat}/K_m^{ATP}$ value for substrate ATP. The greatest changes in the $k_{cat}/K_m^{ATP}$ values were observed in the K27M and K27E mutants with decreases by 1000-fold and 2600-fold, respectively. This lysine interacts with the $\beta$ and $\gamma$ phosphate groups of ATP in AMP kinases (25, 26). Mutations in this lysine in AMP kinases (27) sufficiently perturb the enzyme structure to weaken the interaction between substrates and the enzyme. Similarly in our studies, all mutations at this lysine result in weaker substrate binding. The substitution of lysine with arginine to retain the positive charge resulted in an enzyme that had a $k_{cat}$ value of about half that of the wild-type enzyme. For the K27R mutant, the $k_{cat}/K_m^{ATP}$ value was 60% that of wild-type enzyme, because the K27R mutant retained the positive charge of the side chain, thereby maintaining the electrostatic interactions. The decrease in the $k_{cat}$ value of the K27R mutant must arise from steric differences in the respective side chains of the lysine and arginine. A similar steric interaction in the phosphate-binding loop was found for a K18R mutant of the *E. coli* adenylosuccinate synthetase (7).

When the positive charge of this side chain was removed in the K27M mutant, the $k_{cat}$ value was reduced to 22% that of the wild-type enzyme. The $K_m^{ATP}$ value increased 241-fold, and the $k_{cat}/K_m^{ATP}$ value was reduced 1000-fold. Glutamine substitution for lysine in the *E. coli* AMP kinase (26) also replaces the positively charged lysine sidechain with an uncharged residue. This change causes a decrease in the $k_{cat}/K_m^{ATP}$ similar to our K27M mutant. The *E. coli* enzyme shows a similar decrease in the $k_{cat}/K_m^{UMP}$ for the glutamine substitution for lysine mutant. However, the *Arabidopsis* enzyme shows a slight increase in the $k_{cat}/K_m^{UMP}$ for the K27M mutant. Whether this difference between the glutamine versus methionine mutants is a result of side chain differences or is due to other structural differences between the bacterial and plant enzymes is not clear. Similar results were observed in the K27E mutant in which a decrease in the $k_{cat}/K_m^{ATP}$ is on the same order as with the K27M mutant, yet the $k_{cat}/K_m^{UMP}$ in K27E showed an even greater increase over the wildtype enzyme than did the K27M mutant.

These data indicate that the terminal positive charge of Lys27 is very important for ATP binding. The substitution of this positive charge with an uncharged or negatively charged residue resulted in a significant decrease in ATP binding for the enzyme. The negative charge substitution in the K27E mutant made ATP binding more difficult than noncharge substitution in the K27M mutant. The results also indicated that the structure of the side chain of Lys27 had less effect on ATP binding compared to the positive charge on the side chain. Of those amino acids examined in this study, the Lys27 is much more important for the enzyme to bind ATP.

Unfortunately, two mutants with substitutions of conserved glycine residues at positions 21 and 26 by alanine were unable to be purified for these studies due to degradation. However, this indicated that both glycine residues may be essential to maintain the correct conformation of the protein and function of *Arabidopsis* UMP/CMP kinase as has been found with other enzymes (28).

It's interesting to note that the mutations that had an effect on ATP binding also had an effect on UMP binding. It seems that the binding of one substrate affects the affinity of these mutant enzymes for the other substrate. A similar case has been found in CMP kinase from *E. coli* (29). The *E. coli* CMP kinase shows little overall sequence similarities with other known NMP kinases, but it contained the conserved sequences involved in substrate binding and catalysis. A unique feature not previously observed was that binding of CMP enhanced the affinity of the enzyme for ATP (13).

In this phosphate-binding loop study by site-directed mutagenesis, we have found that the phosphate-binding loop in *Arabidopsis* UMP/CMP kinase plays a role in ATP binding and enzyme catalysis. It would be interesting to see how the conserved residues in the phosphate-binding loop interact with the phosphoryl groups of ATP in the 3-D structure of the enzyme. This would provide a structural basis for the alterations of kinetic parameters by the site-directed mutagenesis in this report. The 3-D structure also would provide insight into how the base specificity is determined, which would be a guide to change substrate specificity by site-directed mutagenesis.

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