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Mutagenesis of apyrase conserved region 1 alters the nucleotide substrate specificity

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Abbreviations: NTPDase, Nucleotide triphosphate diphosphohydrolase

Two apyrases having different substrate specificity, MP67 and *Mp*APY2, are present in *Mimosa pudica*. The substrate specificity of MP67 is quite high against ADP, and is distinct from any other apyrase. This might be attributed to the nucleotide binding motif (DXG) in apyrase conserved region 1. We performed a single amino acid substitution at position X in the motif. The ratio of the velocity of ATP/ADP hydrolysis was higher (approximately 1) for the S63A-MP67 mutant than for wild type-MP67 (0.19). Binding affinity for ADP of A75S-*Mp*APY2 mutant was increased to a level higher than that of the wild type *Mp*APY2. Thus, the residue at position X in the DXG motif plays an important role in determining nucleotide preference.

Apyrase [nucleotide triphosphate diphosphohydrolase (NTPDase), EC 3.6.1.5] is an enzyme found in all prokaryotes and eukaryotes.[1] All apyrases contain 5 highly conserved apyrase conserved regions (ACR1-5), and exhibit low substrate specificity in the presence of divalent metal ion, i.e., they hydrolyze most nucleoside triphosphates (NTPs) and diphosphates (NDPs).[2-9]

We recently reported the presence of two apyrases, MP67 and *Mp*APY2, in *Mimosa pudica*. The MP67 is biochemically distinct from other apyrases, and demonstrates substantially higher substrate specificity for ADP than for ATP. In general, animal and monocot plant apyrases are characterized by the ATP/ADP hydrolysis velocity ratio (K_{sh}) > 1, whereas in dicot plants, the K_{sh} is typically < 1, with the standard range of 0.4 to 1.8.[10] The specific activity of MP67 for the hydrolysis of ADP is extremely higher than for the hydrolysis of ATP.[7] The biochemical uniqueness of MP67 can be further demonstrated by differences in sensitivity to inhibitors. Apyrases are generally known to be insensitive to inhibitors of P-type, F-type and V-type ATPases; however, MP67 is sensitive to inhibitors of P-type ATPases.[7,10,11] Moreover, although the MP67 enzyme contains the typical apyrase conserved region ACR1-5, comparison of the primary sequences showed that MP67 and *Mp*APY2 share only 45% identity. Phylogenetic analyses showed that MP67 is a noncanonical apyrases.[7] Among the conserved regions, ACR1 and ACR4 are the most important with respect to nucleotide binding. Both ACR1 and ACR4 contain prominent "DXG" motifs, where X denotes any amino acid. Structurally, the DXG motif found in the apyrase conserved regions is quite similar to that found in members of the actin/hsp70/sugar kinase superfamily.[12,13] Mutagenesis data show that the DXG motifs are involved in substrate recognition and binding, hydrolysis and cation coordination.[12,14] The importance of the DXG motif in apyrases was established through mutation of the Asp and Gly residues, which has been shown to result in inactivation of human NTPDase3.[12,13] Almost all apyrases contain an Ala residue at the X position in the DXG motif, thought the importance of Ala residue at the X position has not been evaluated. Using site-directed mutagenesis, we demonstrate here that the amino acid residue at position X in the DXG motif is important for substrate recognition, and is especially important in determining substrate preference.

To assess whether the particular substrate specificity of MP67 is determined by the primary structure of the protein, sequences encompassing regions most likely involved in nucleotide binding (i.e., ACR1 and ACR4) were aligned with the ACR1 and ACR4 sequences of apyrases from various other species (**Table 1**). The X position in the DXG motif in most of the apyrase ACR1 sequences examined is occupied by Ala (458 out of 460, data not shown). However, human 70 kDa lysosomal apyrase-like protein (LALP70, NTPDase4, UDPase)[15,16] and LALP1 (NTPDase7)[17] are exceptions; these proteins contain a Cys residue at the X position. In addition, like MP67, the yeast NDPase, which has high activity for NDP, contains a Ser residue at the X

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Table 1. Multiple sequence alignment of apyrase ACRs

Apyrase	ACR1	ACR4	GenBank ID
MP67	62 **DSG**STGT	209 **DLG**GGSVQ	BAK78977
MpAPY2	74 **DAG**SSGS	220 **DLG**GGSVQ	BAK78982
*At*APY1	76 **DAG**SSGS	222 **DLG**GGSVQ	AAF00612
Potato apyrase	51 **DAG**STGS	197 **DLG**GGSVQ	AAB02720
Human CD39	54 **DAG**SSHT	213 **DLG**GASTQ	P49961
Mouse CD39	54 **DAG**SSHT	212 **DLG**GASTQ	P55772
Human LALP70	94 **DCG**SSGS	271 **DMG**GVSTQ	Q9Y227
Human LALP1	89 **DCG**SSGS	258 **DMG**GASLQ	Q9NQZ7
S. cerevisiae GDPase	98 **DAG**STGS	245 **DLG**GGSTQ	AAA34656
S. stipitis NDPase	15 **DSG**SSGS	185 **DMG**GASTQ	EAZ63008

The conserved nucleotide binding motif DXG is underlined and the residue at position X is indicated in bold. The amino acid sequences of apyrase conserved regions 1 and 4 (ACR1 and ACR4) from MP67, *Mp*APY2 (*Mimosa pudica* apyrase2), *At*APY1 (*Arabidopsis thaliana* apyrase 1), potato apyrase (*Solanum tuberosum* apyrase), human CD39 (*Homo sapiens* NTPDase1), mouse CD39 (*Mus musculus* NTPDase1), human LALP70 (NTPDase4), human LALP1 (NTPDase7), *S. cerevisiae* GDPase (*Saccharomyces cerevisiae*) and *S. stipites* NDPase (*Saccharomyces stipitis*) are shown.

position, though *S. cerevisiae* GDPase contains an Ala residue at the X position.[18,19] In ACR4, most apyrases contain a Leu residue at the X position (396 out of 460, data not shown), though apyrases from several species contain a Met or other residue at the X position. MP67 contain a well-conserved Leu residue at the X position in ACR4. Thus we considered that the X position in ACR1 might contribute to the characteristics in nucleotide hydrolysis of MP67. We therefore examined the role of the X-residue in the DXG motif in ACR1 of MP67 and *Mp*APY2 using site-directed mutagenesis substitution of the Ala and Ser residues, respectively. Ala substitution was done because it is the most frequently occurring residue at the X position in the DXG motif of other ACR1s. In addition, the Ala normally located at the X position in *Mp*APY2 was substituted with Ser, which is found at the X position in MP67. Both ATP and ADP were used as substrates to measure the hydrolytic activity of the recombinant proteins (**Fig. S1**). The hydrolysis of ATP and ADP was analyzed by presenting Lineweaver-Burk plots to determine whether the observed changes in hydrolytic activity of the mutant enzymes were due to the changes in substrate affinity or in the hydrolysis velocity. As reported previously, the wild type (WT)-MP67 hydrolyzed ADP quickly, and hydrolyzed ATP slowly. However, the hydrolysis velocity of S63A-MP67 mutant for ADP decreased greatly and the velocity for ATP increased, and was reached to the same level (**Fig. 1A and B**). On the other hand, the hydrolysis velocity of A75S-*Mp*APY2 mutant for ATP was decreased as compared with WT-*Mp*APY2, though the velocity for ADP was not significantly changed (**Fig. 1C and D**). Thus, the nucleotide hydrolysis velocities for ATP and ADP were reversed slightly by changing a single amino acid residue. As summarized in **Figure 2**, the V_{max} value for ADP hydrolysis by the S63A-MP67 mutant dropped to the same level as for the hydrolysis of ATP. And no significant change in the V_{max} value of A75S-*Mp*APY2 mutant for ADP was observed after the amino acid substitution, whereas that for ATP was decreased. Our data showed that the nucleotide hydrolysis properties of the S63A-MP67 mutant are different from those of WT-MP67, and that the K_{sh} for this mutant is almost 1, compared with 0.19 for WT-MP67. In contrast, we found that the K_{sh} value for WT-*Mp*APY2 is 1.20, and that for A75S-*Mp*APY2 mutant is 0.72. Thus, our data let us consider that the substation of amino acid at X position in ACR1 affects nucleotide hydrolysis property of apyrases. As reported in the previous work, the K_m value of WT-MP67 was lower for ADP than for ATP, and the K_m values of WT-*Mp*APY2 for ATP and ADP were the same level (**Fig. 2B**). Substitution of Ser for Ala decreased the K_m value for the S63A-MP67 mutant for ATP to 114.8 μM, while the K_m for ADP increased to 65.5 μM. Notably, the K_m values for the A75S-*Mp*APY2 mutant indicated that there was a change in the affinity for ATP and ADP, and that the substitution led to a reverse in substrate specificity. Thus, since both the K_m and V_{max} differed from the values for the WT enzyme, we concluded that the residue in position X in the DXG motif of ACR1 does influence substrate affinity and reaction velocity during hydrolysis of nucleotide substrates.

Consistent with our finding, yeast NDPase also contain a Ser residue at position X, and preferentially hydrolyzes NDP. LALP70 and LALP1 contain a Cys residue at position X, but these enzymes show different substrate specificity; LALP70 preferentially hydrolyzes ADP rather than ATP, while the hydrolysis of ATP by LALP1 is quicker than for ADP.[16,17] Cys is structurally similar to Ser, though it possesses the reactive thiol side chain. Thus, the substrate specificity is not attributed either to the hydrophobicity or to the structural feature of a single amino acid residue at X position but might be strictly regulated by the higher structure of apyrase. X-ray structural analyses revealed that ACR1 and ACR4 in rat apyrase (*Rn*NTPDase2) and *Legionella pneumophila* apyrase (*Lp*1NTPDase) are involved in binding the β- and γ-phosphates of ATP.[20,21] In *Rn*NTPDase2, divalent metal ion is thought to function as a catalyst by polarizing one of the P-O bonds of the terminal phosphate.[21] The importance of correct coordination of the cofactor is underpinned by the fact that mutation of residues involved in divalent metal ion binding (i.e., D45 in ACR1, D201 in ACR4, and W436 in ACR5) can result in loss of activity[13,14,22,23] or alteration of substrate and/or cofactor specificity and affinity.[14,24] Those three residues form an octahedral coordinated geometry for Ca^{2+} ion in conjunction with the β- and γ-phosphate groups of nucleotides.[20] Therefore, we suggested that X residue in DXG motif may participate in the selectivity of the nucleotide which mediated divalent metal ion indirectly. Further structural analyses are needed to determine whether the position X in the DXG motif plays a critical role in the substrate recognition.

Material and Methods

Site-directed mutagenesis. cDNA clones encoding the protein coding regions, but lacking the transmembrane domain-coding regions of *MP67* (GenBank ID: AB600992) and *MpAPY2*

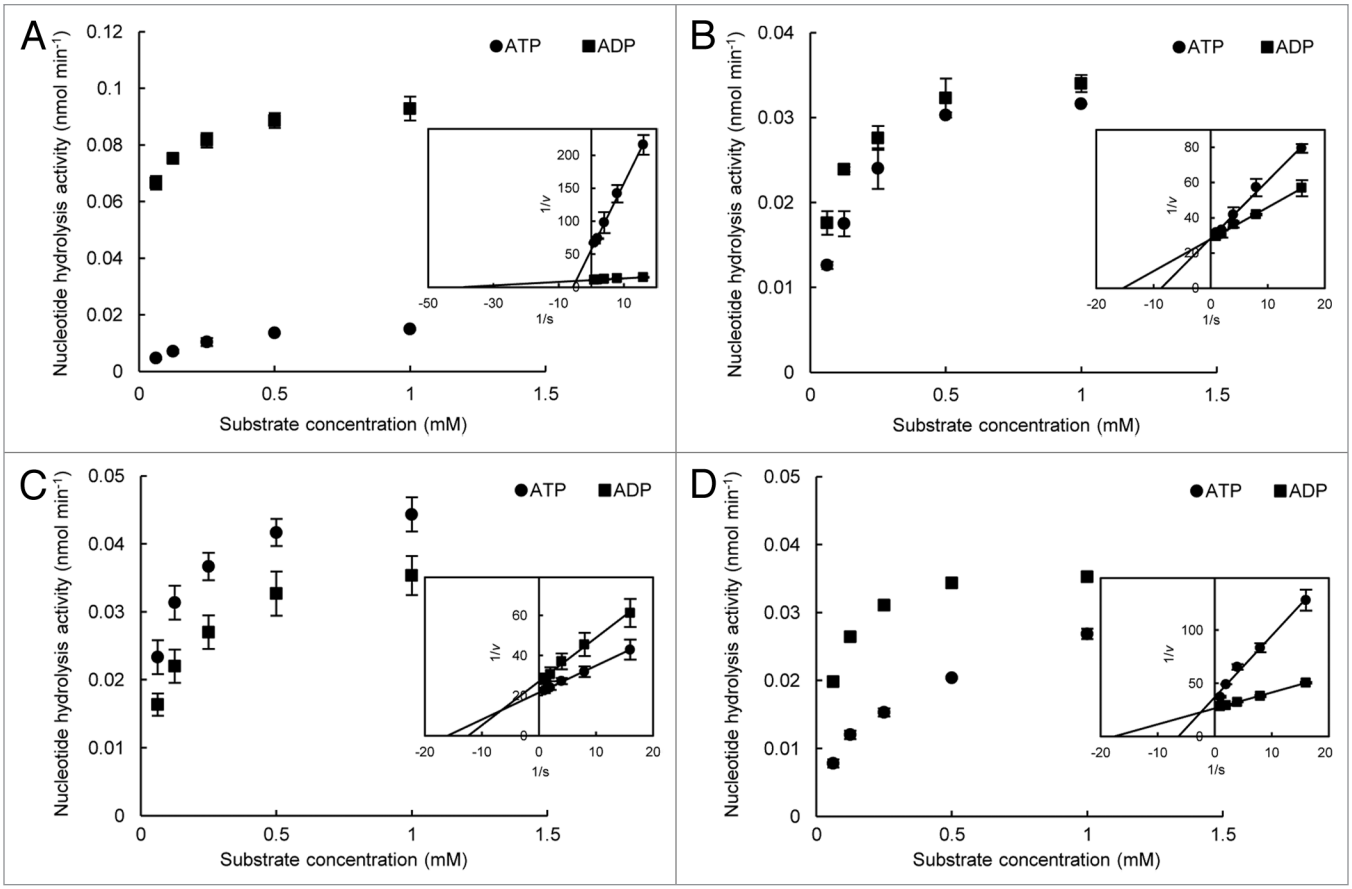

Figure 1. Nucleotide hydrolysis velocity of WT and mutant apyrases. Enzymatic parameters were estimated by initial velocity vs. substrate concentration for ATP and ADP with WT- MP67 (**A**), S63A-MP67 mutant (**B**), WT-*Mp*APY2 (**C**) and A75S-*Mp*APY2 mutant (**D**). K_m and V_{max} values were estimated according to a double reciprocal plot (inset) and linear regression analysis. Results are means ± SE (n = 6).

(GenBank ID: AB600997) were ligated into the pCold I expression vector (TaKaRa) as described previously,[7] and were used as templates for site-directed mutagenesis. Serine-63 of MP67 and Ala-75 of *Mp*APY2 were substituted to Ala and Ser (S63A-MP67 and A75S-*Mp*APY2), respectively, using the primer pairs (mismatched nucleotides are underlined): 5'-GAT ATT GGA CGC GGG GAG CAC AGG-3' and 5'-CCT GTG CTC CCC GCG TCC AAT ATC-3' for S63A-MP67, and 5'-GTT ATC TTT GAT AGC GGT AGT TCT GG-3' and 5'-CCA GAA CTA CCG CTA TCA AAG ATA AC-3' for A75S-*Mp*APY2. Site-directed mutagenesis with these primers was performed using a Quickchange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Mutations in the resulting clones were confirmed by DNA sequencing using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Purification of recombinant proteins. Genes encoding MP67, S63A-MP67, *Mp*APY2 and A75S-*Mp*APY2 inserted in pCold I were used to transform *Escherichia coli* BL21 (DE3), and recombinant proteins were purified from both the supernatant and inclusion bodies of positive transformant cultures using His-Select nickel affinity columns (Sigma-Aldrich), as described previously.[7] Purified recombinant proteins were dialyzed overnight at 4°C against 20 mM TRIS-HCl/300 mM NaCl.

Nucleotide hydrolyzing activity assay. Purified recombinant protein (600 ng) was added to an assay mixture consisting of 60 mM KCl, 2 mM $CaCl_2$, and 40 mM TRIS-HCl (pH 7.5). Hydrolysis of different nucleotide substrates was initiated by adding substrate to a final concentration of 1 mM, and the mixture was incubated for 30 min at 25°C. The reaction was terminated by addition of acid malachite green solution and inorganic phosphates were subsequently determined using a colorimetric procedure[25] with slight modifications, as described previously.[26] Values for V_{max} and K_m were determined for hydrolysis of ATP and ADP from Lineweaver-Burk plots.[27] The statistical significance of differences was analyzed using the Student's t-test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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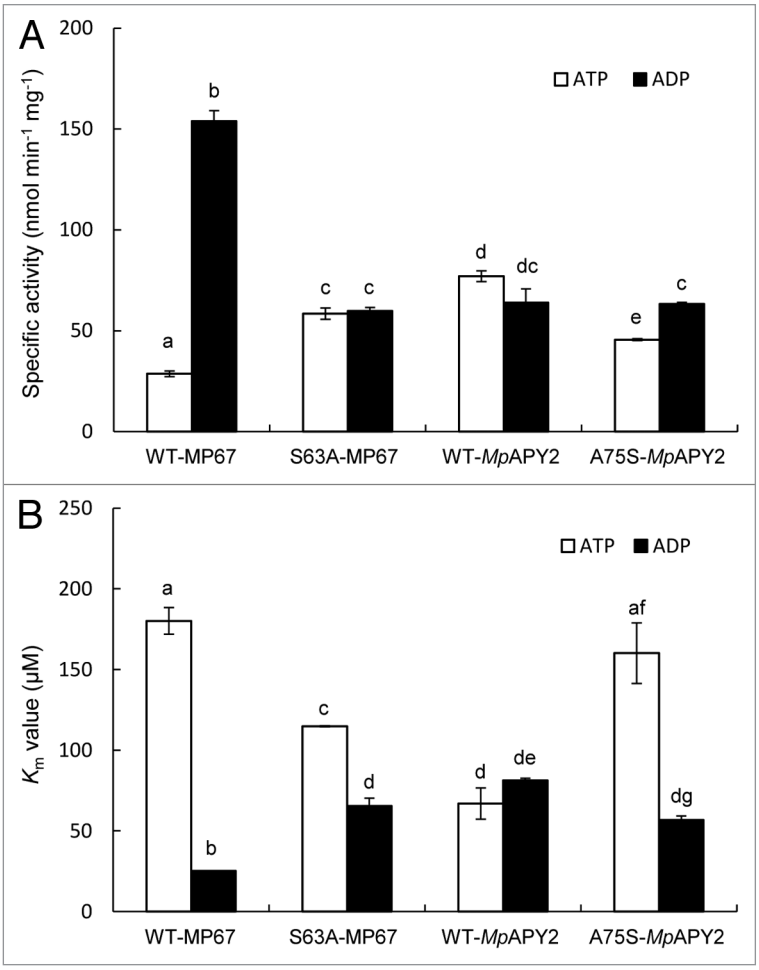

Figure 2. Comparison of V_{max} and K_m values of WT and mutant apyrases. Specific activity (**A**) and K_m values (**B**) for ATP (open bar) and ADP (solid bar) were represented. Results are means ± SE (n = 6). Different letters above the bars indicate mean values that were significantly different from other ($P < 0.05$).

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