

RESEARCH ARTICLE

A remote surface loop modulates core structure and cold activity in phosphopantetheine adenylyltransferase

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Abstract

Phosphopantetheine adenylyltransferase (PPAT), a key enzyme in the universal Coenzyme A biosynthetic pathway, is essential for cellular metabolism. However, the adaptive mechanisms of PPAT in psychrophilic (cold-adapted) organisms remain poorly understood. Here, we characterize PPAT from the psychrophilic methanotroph *Methylocapsa palsarum* (MpaPPAT). Sequence analysis identified a unique five-amino-acid insertion (SCRLS) within a surface-exposed loop, a feature conserved among psychrophilic homologues. To investigate its function, we determined the crystal structures of wild-type (WT) MpaPPAT and a loop-deletion mutant (MpaPPAT(Δ67–71)) and performed comparative biochemical analyses. Structurally, MpaPPAT forms a dimer-of-trimers hexamer. Biochemically, WT MpaPPAT maintains high catalytic activity at low temperatures (10–20 °C), whereas the MpaPPAT(Δ67–71) mutant exhibits impaired cold activity. The mutant structure reveals that the deletion of the distant surface loop induces a long-range allosteric change, resulting in a dual impairment: 1) a stabilization and rigidification (“clamping”) of the central α-helix 4 (H4) at the hexameric core interface, and 2) a dramatic shift in the central pore’s electrostatic potential from positive (WT) to negative (mutant). Our findings reveal that the SCRLS insertion is a critical allosteric modulator that provides a sophisticated dual mechanism for enzymatic cold adaptation. It maintains the conformational flexibility of the hexameric core, preventing the “clamping” effect, and simultaneously ensures a positively charged central channel to electrostatically steer negatively charged substrates (ATP and phosphopantetheine) into the active site, thereby overcoming the kinetic challenges of a low-temperature environment.

1. Introduction

Coenzyme A (CoA), the principal cofactor in several biosynthetic and degradative pathways, is a universal acyl group carrier in all living cells [1,2]. CoA plays essential

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roles in metabolic processes, such as the citric acid cycle, fatty acid biosynthesis, and lipopolysaccharide biosynthesis [3]. In bacteria, CoA is required for the biosynthesis of membrane lipids, peptidoglycan, teichoic acids in Gram-positive bacteria, and lipid A in Gram-negative bacteria [4–9].

The biosynthesis of CoA proceeds through a five-step enzymatic pathway that utilizes pantothenate (vitamin B5), cysteine, and ATP as precursors. This pathway begins with the phosphorylation of pantothenic acid to form 4'-phosphopantothenate. Subsequently, 4'-phosphopantothenate condenses with cysteine to produce 4'-phosphopantothienoylcysteine, which is a reaction catalyzed by phosphopantothienoylcysteine synthetase. This intermediate, 4'-phosphopantothienoylcysteine, is then decarboxylated by phosphopantothienoylcysteine decarboxylase to yield 4'-phosphopantetheine. In the penultimate step, phosphopantetheine adenyllyltransferase (PPAT) converts 4'-phosphopantetheine to dephospho-CoA. As the final step, dephospho-CoA is then converted to CoA by the action of CoA synthetase.

PPAT is produced from the *coaD* gene and requires magnesium to transfer an adenyllyl group from ATP to 4'-phosphopantetheine, creating dephospho-CoA (dPCoA) and pyrophosphate (Fig 1). In studies of *Escherichia coli* PPAT (EcoPPAT), substantial accumulation of the metabolic intermediate 4'-phosphopantetheine was observed, indicating that PPAT does not continuously run at full capacity but instead responds to cellular conditions in controlling intracellular CoA concentrations through feedback mechanisms [10]. This finding suggests that PPAT serves as a rate-limiting step in the pathway. This regulatory system influences the re-use of 4'-phosphopantetheine that comes from CoA cleavage through phosphodiesterase or from releasing the 4'-phosphopantetheinyl cofactor associated with the acyl carrier protein [10,11]. In addition to PPAT, pantothenate kinase, the first enzyme in the CoA biosynthetic pathway, contributes to rate control through competitive inhibition, as CoA and its acyl thioesters act as competitive inhibitors of ATP binding [12–15].

While researchers have characterized PPAT structure and biochemistry in standard model organisms, such as *E. coli*, far less is known about how this enzyme functions in bacteria with specialized metabolism. *Methylocapsa palsarum* represents one such organism. Isolated from collapsed palsa soil in Norway's subarctic region under cold conditions [16], *M. palsarum* is a psychrophilic bacterium, making it valuable for examining how enzymes and metabolic pathways adapt to low temperatures.

Enzymes adapted to cold environments frequently possess enhanced structural flexibility, particularly in surface-exposed loops, to compensate for reduced thermal energy [17]. Classic examples, such as psychrophilic α -amylase and trypsin, exhibit elongated or more flexible loops compared to their mesophilic counterparts, which are linked to their high catalytic efficiency at low temperatures [18,19]. Preliminary sequence alignment of PPAT from *M. palsarum* (MpaPPAT) with its homologues revealed a unique five-amino-acid insertion (SCRLS) in a region predicted to form a surface-exposed loop (Fig 2A). This observation led us to hypothesize that this insertion may represent a specific structural adaptation to cold environments. However, it was unclear whether this insertion indeed forms a flexible loop and what specific mechanistic role it plays in catalysis at low temperatures.

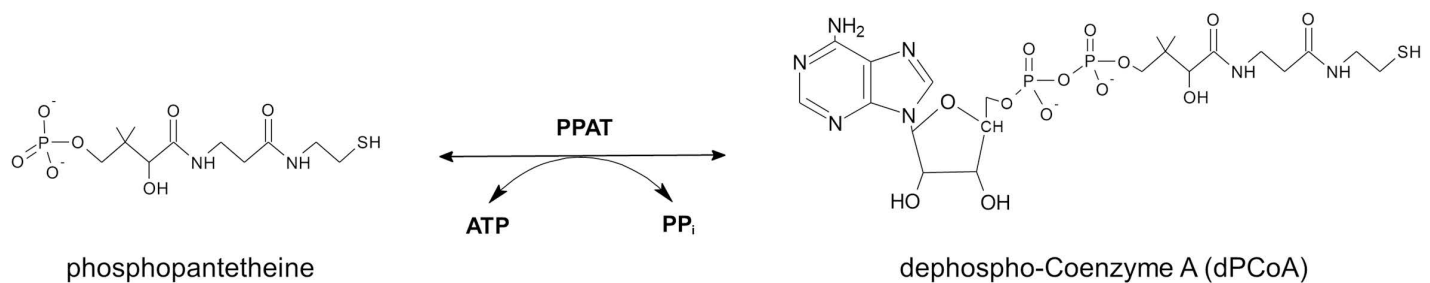


Fig 1. Scheme of the penultimate step in the CoA biosynthetic pathway catalyzed by phosphopantetheine adenylyltransferase (PPAT). PPAT reversibly transfers an adenylyl group from ATP to 4'-phosphopantetheine, producing 3'-dephospho-coenzyme A (dPCoA) and pyrophosphate (PP_i).

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In this study, we combine X-ray crystallography, steady-state kinetics, and biophysical analyses to characterize *MpaPPAT*. We determined the crystal structures of wild-type *MpaPPAT* and a loop-deletion mutant and assessed their kinetic properties. Our results reveal that the SCRLS insertion forms a critical, cold-adapted surface loop that functions as an allosteric modulator, remotely controlling the flexibility of the hexameric core to ensure high catalytic activity at low temperatures.

2. Materials and methods

2.1. Cloning and site-directed mutagenesis

The gene encoding *MpaPPAT* from *M. palearum* was codon-optimized for *Escherichia coli* expression, synthesized (Bioneer, Korea), and subcloned into the pET28a vector (Novagen EMD Biosciences, Inc., Merck) using NdeI and XhoI restriction sites. To construct the loop deletion mutant (*MpaPPAT*(Δ67–71)), site-directed mutagenesis was performed using an overlap extension PCR strategy. In the first step, two separate PCR fragments were amplified from the wild-type plasmid: an N-terminal fragment using primers Δ*MpaPPAT*_F1 and Δ*MpaPPAT*_R1 (the reverse overlap primer), and a C-terminal fragment using primers Δ*MpaPPAT*_F2 (the forward overlap primer) and Δ*MpaPPAT*_R2. These two purified fragments, which overlap at the deletion site, were then used as a template in a subsequent PCR reaction using only the outer primers (Δ*MpaPPAT*_F1 and Δ*MpaPPAT*_R2) to generate the full-length mutated construct. The final PCR product was verified using DNA sequencing. The primers used for mutagenesis are listed in [S1 Table](#).

2.2. Protein expression and purification

The recombinant pET28a plasmids containing the genes encoding hexahistidine-tagged *MpaPPAT* and *MpaP-PAT*(Δ67–71) were introduced into *E. coli* BL21 (DE3) cells. Cultures were grown in Luria-Bertani (LB) medium supplemented with 50 μg/mL kanamycin at 37 °C until the optical density at 600 nm (OD₆₀₀) reached 0.5. Protein expression was induced by adding 0.5 mM isopropyl-β-D-1-thiogalactoside (IPTG), and culture incubation was continued at 25 °C for 20 hours. Following induction, cells were collected by centrifugation (6000 rpm, 30 min), resuspended in lysis buffer (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 20 mM Imidazole), and then lysed by ultrasonication. The soluble fraction was separated by centrifugation (16,000 rpm, 40 min) and loaded onto a pre-equilibrated opentop Ni-NTA column to remove unwanted proteins using the 20 column volumes of lysis buffer. The bound proteins were eluted using elution buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, and 300 mM Imidazole). Finally, the protein was further purified and buffer-exchanged by size-exclusion chromatography (SEC) performed using a HiLoad 16/600 Superdex 200-pg column (Cytiva, Marlborough, MA, USA) equilibrated with 20 mM Tris-HCl (pH 8.0) and 200 mM NaCl. The purified *MpaPPAT* and *MpaP-PAT*(Δ67–71) were concentrated using an Amicon Ultra centrifugal filter 10 kDa MWCO (Merck, Darmstadt, Germany) to final concentrations of approximately 20 mg/mL and 33 mg/mL, respectively.

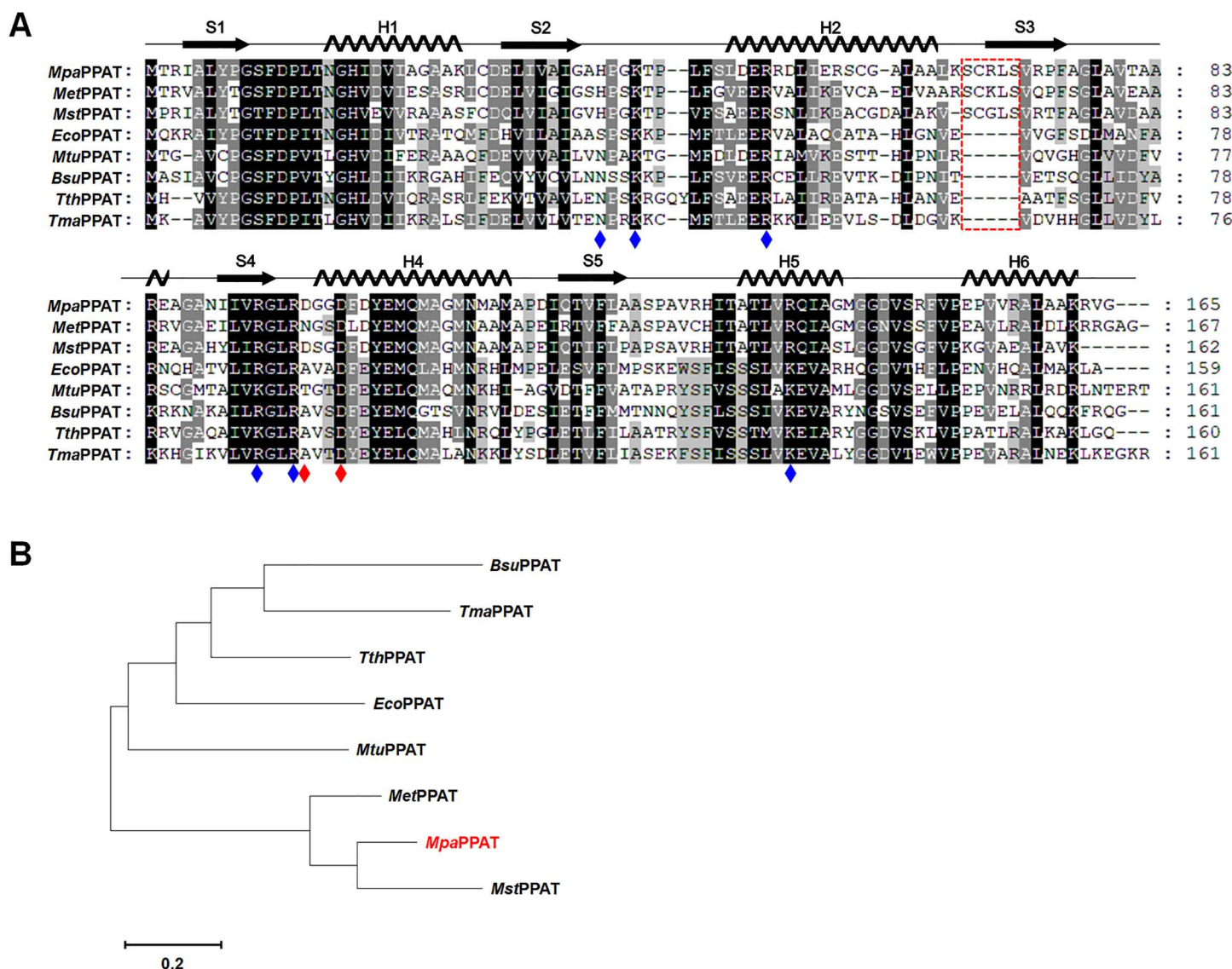


Fig 2. Multiple sequence alignment and phylogenetic analysis of *MpaPPAT*. (A) Multiple sequence alignment of *MpaPPAT* with its homologues: Psychrophilic homologues, including *MetPPAT* (*Methylocella tundrea*) and *MstPPAT* (*Methyloferula stellata*); Mesophilic homologues, including *EcoPPAT* (*Escherichia coli*), *MtuPPAT* (*Mycobacterium tuberculosis*), and *BsuPPAT* (*Bacillus subtilis*); and Thermophilic homologues, including *TthPPAT* (*Thermus thermophilus*) and *TmaPPAT* (*Thermotoga maritima*). Highly conserved residues are highlighted in black, and partially conserved residues are shown in gray. The unique surface loop insertion (SCRLS in *MpaPPAT*) found in psychrophilic homologues is boxed in red. Positively and negatively charged residues lining the central funnel are indicated by blue and red diamonds, respectively. (B) Phylogenetic analysis of *MpaPPAT* homologues. The tree was constructed using the unrooted Neighbor-Joining method based on the alignment of the selected protein sequences.

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2.3. Crystallization and X-ray data collection

The *MpaPPAT* and *MpaPPAT*(Δ67–71) mutant were used for crystallization experiments. Preliminary crystallization screening was conducted using the sitting-drop vapor-diffusion method in 96-well crystallization plates using a Mosquito crystallization robot (TTP LabTech, Melbourn, UK). Commercially available crystallization kits were used, including MCSG 1T to 4T (Anatrace, Maumee, USA), Morpheus (Molecular Dimensions, Rotherham, UK), SaltRx, and Index (Hampton

Research, Aliso Viejo, CA, USA). In each well, 400 nL protein solution was mixed with 400 nL reservoir solution and allowed to equilibrate against 80 μ L of the reservoir solution. To obtain optimized crystals, the hanging-drop vapor diffusion method was employed. Crystals of wild-type *Mpa*PPAT were obtained in a reservoir solution containing 0.2 M $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ (pH 6.4) and 2.9 M sodium chloride, while *Mpa*PPAT(Δ 67–71) crystals were grown in a solution composed of 2.2 M sodium chloride and 0.1 M Tris-HCl (pH 8.5). Crystallization trials were set up in a 24-well VDX™ plate with sealant (Hampton Research, Aliso Viejo, CA). A 500 μ L reservoir solution was pipetted into each well, and a droplet was formed by mixing 1.5 μ L of protein solution with 1.5 μ L of the reservoir solution. Diffraction data were collected at the 5C beamline of Pohang Light Source-II (PLS), Korea. A single crystal of *Mpa*PPAT and *Mpa*PPAT(Δ 67–71) was carefully selected and mounted. The wild-type *Mpa*PPAT crystal was cryoprotected using Paratone-N-oil, whereas the *Mpa*PPAT(Δ 67–71) crystal was mounted directly without additional cryoprotection. A total of 360 diffraction images were recorded, and each data set was indexed, integrated, and scaled using the XDS software package.

2.4. Structure determination and refinement

The crystal structures of apo wild-type *Mpa*PPAT and *Mpa*PPAT(Δ 67–71) were determined at 2.11 Å and 2.63 Å resolution, respectively. The molecular replacement method was employed using the Molrep program within the CCP4i suite [20]. For *Mpa*PPAT, the structure of the AlphaFold-based homology model was used as a search model [21]. The refined wild-type structure subsequently served as the search model for the *Mpa*PPAT(Δ 67–71) structure. Iterative cycles of model building and refinement were conducted using Coot and phenix.refine [22–24]. Final structures were validated using MolProbity and deposited in the Protein Data Bank (PDB) under accession codes 9XWD for (*Mpa*PPAT) and 9XWQ for (*Mpa*PPAT(Δ 67–71)), respectively. The detailed statistics for data collection and structural refinement are summarized in S2 Table. All structural figures were prepared using PyMOL [25].

2.5. Reverse-direction steady-state assay for measurement of transferase activity

To evaluate enzymatic activity, we employed a reverse-direction coupled assay based on hexokinase and glucose-6-phosphate dehydrogenase (G6PD) to quantify ATP production [1]. In this assay, ATP generated by PPAT is consumed by hexokinase, and the subsequent G6PD-catalyzed reaction produces NADPH, which is monitored spectrophotometrically at 340 nm [26,27]. To assess temperature-dependent activity, the experiment was structured into two sequential steps. The first step exposed the enzyme reaction to different temperatures, while the second step was performed at room temperature.

For the initial stage, a 97 μ L reaction mixture was prepared containing 0.025 μ M dPCoA, 2 mM sodium pyrophosphate, and 250 nM enzyme (*Mpa*PPAT or *Mpa*PPAT(Δ 67–71)) in 20 mM Tris-HCl buffer (pH 8.0) with 200 mM NaCl. Reactions were incubated at temperatures ranging from 10 to 60 °C for 1 min, then heat-inactivated at 92 °C for 2 min, and cooled at 4 °C for 3 min. In the second phase, 3 μ L of a coupling mixture was added to each reaction (final volume 100 μ L). The final concentrations of the added components were 1 mM NADP^+ , 5 mM MgCl_2 , 5 mM glucose, 5 U of hexokinase, and 5 U of G6PD. After incubation at room temperature (2 min), the ATP produced in the first step was converted to ADP by hexokinase, yielding glucose 6-phosphate. This product was subsequently oxidized to 6-phosphogluconolactone with concomitant reduction of NADP^+ to NADPH. NADPH formation was monitored by measuring absorbance at 340 nm.

2.6. Analytical ultracentrifugation assay

The oligomerization state of *Mpa*PPAT in solution was examined using a ProteomeLab XL-A analytical ultracentrifuge (Beckman Coulter, Inc.). Protein samples were prepared at 1 mg/mL in 20 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl, and 400 μ L of each sample was loaded into the cell. The samples were centrifuged at 42,000 rpm at 20 °C, and absorbance at 280 nm was recorded at 5 min intervals. The partial specific volume (v -bar) and buffer density/viscosity were calculated using SEDNTERP (Ver. 3.03), and data were plotted using GUSI software (Ver. 1.4.2). The resulting

sedimentation velocity profiles were analyzed using SEDFIT (Ver. 16.36) to generate the continuous sedimentation coefficient distribution, $c(s)$.

3. Results and discussion

3.1. Sequence analysis of MpaPPAT reveals a unique loop insertion associated with psychrophilic homologues

To investigate the structural characteristics of *MpaPPAT* and its relationship to other known PPAT enzymes, we performed a homologue search and generated a multi-sequence alignment (MSA) (Fig 2A). The MSA revealed significant overall sequence identity and similarity, confirming *MpaPPAT* belongs to the phosphopantetheine adenylyltransferase family. As highlighted in Fig 2A, *MpaPPAT* retains the highly conserved core structural elements of the PPAT family, including the regions corresponding to β -strands (S1 and S4) and α -helices (H1, H4, and H5). Furthermore, key residues essential for catalysis and substrate binding, such as the highly conserved histidine and arginine residues (e.g., H18 and R91 in *EcoPPAT*) involved in binding ATP and 4'-phosphopantetheine, were conserved in *MpaPPAT* [28–30]. This conservation suggests a shared catalytic mechanism.

However, a notable difference was identified in the region connecting H2 and S3, which is generally not conserved across PPAT homologues. While many characterized mesophilic and thermophilic homologues (e.g., *E. coli* [31], *Mycobacterium tuberculosis* [32], *Bacillus subtilis* [33], *Thermus thermophilus* [34], and *Thermotoga maritima*) lack a distinct insertion in this region, *MpaPPAT* possesses a unique five-amino-acid insertion (SCRLS). Intriguingly, phylogenetic analysis and multiple sequence alignment revealed that this feature is not a random phenomenon. Homologues from phylogenetically related psychrophilic or soil bacteria, specifically *Methylocella tundrae* (*MetPPAT*) and *Methyloferula stellata* (*MstPPAT*), also possess a corresponding five-amino-acid insertion at this locus (Fig 2A and 2B). However, the specific sequence 'SCRLS' is unique to *MpaPPAT*. This observation is noteworthy, as this insertion is located within a variable region predicted to form an exposed surface loop. Such features are often linked to enhanced structural flexibility, a common strategy for enzymatic cold adaptation that typically involves the elaboration of loops or a reduction in intramolecular interactions [19,30]. This enhanced flexibility is thought to provide the necessary conformational freedom to maintain efficient catalytic activity at low temperatures, thereby compensating for the reduced thermal energy. Based on this sequence analysis, we hypothesize that the SCRLS insertion in *MpaPPAT* forms an extended, flexible surface loop that contributes to its adaptation and function at low temperatures [35].

3.2. Crystal structure determination and overall fold of MpaPPAT

To test our hypothesis that the SCRLS insertion in *MpaPPAT* forms a unique structural element, we determined the crystal structure of apo-*MpaPPAT* at 2.11 Å resolution by X-ray crystallography (S2 Table). Phasing was successfully achieved by molecular replacement using an AlphaFold-based homology model [21]. Iterative rounds of model building and refinement yielded a final structure with R_{work} and R_{free} values of 25% and 29%, respectively. Structural analysis reveals that the *MpaPPAT* monomer adopts a dinucleotide-binding fold characterized by a central five-stranded parallel β -sheet, which is characteristic of the cytidylyltransferase fold, a variant of the Rossmann-like superfamily [31]. The β -sheet displays a strand topology of S3-S2-S1-S4-S5 and is surrounded by α -helices: four helices (H1, H2, H5, and H6) on one side and two helices (H3 and H4) on the opposite side (Fig 3A). The C-terminus of *MpaPPAT* contains a short 3_{10} -helix preceding H5, which forms a 3_{10} -helix pair characteristic of PPAT enzymes [28].

3.3. MpaPPAT assembles into a dimer-of-trimers hexamer

In its quaternary structure, *MpaPPAT* assembles into a hexamer, which adopts a 'dimer-of-trimers' arrangement, which is observed in the PPAT enzyme family (Fig 3B). The main oligomeric interface is formed by the packing of the C-terminal helices, particularly H4 and H5, from adjacent monomers. However, a notable conformational heterogeneity is observed

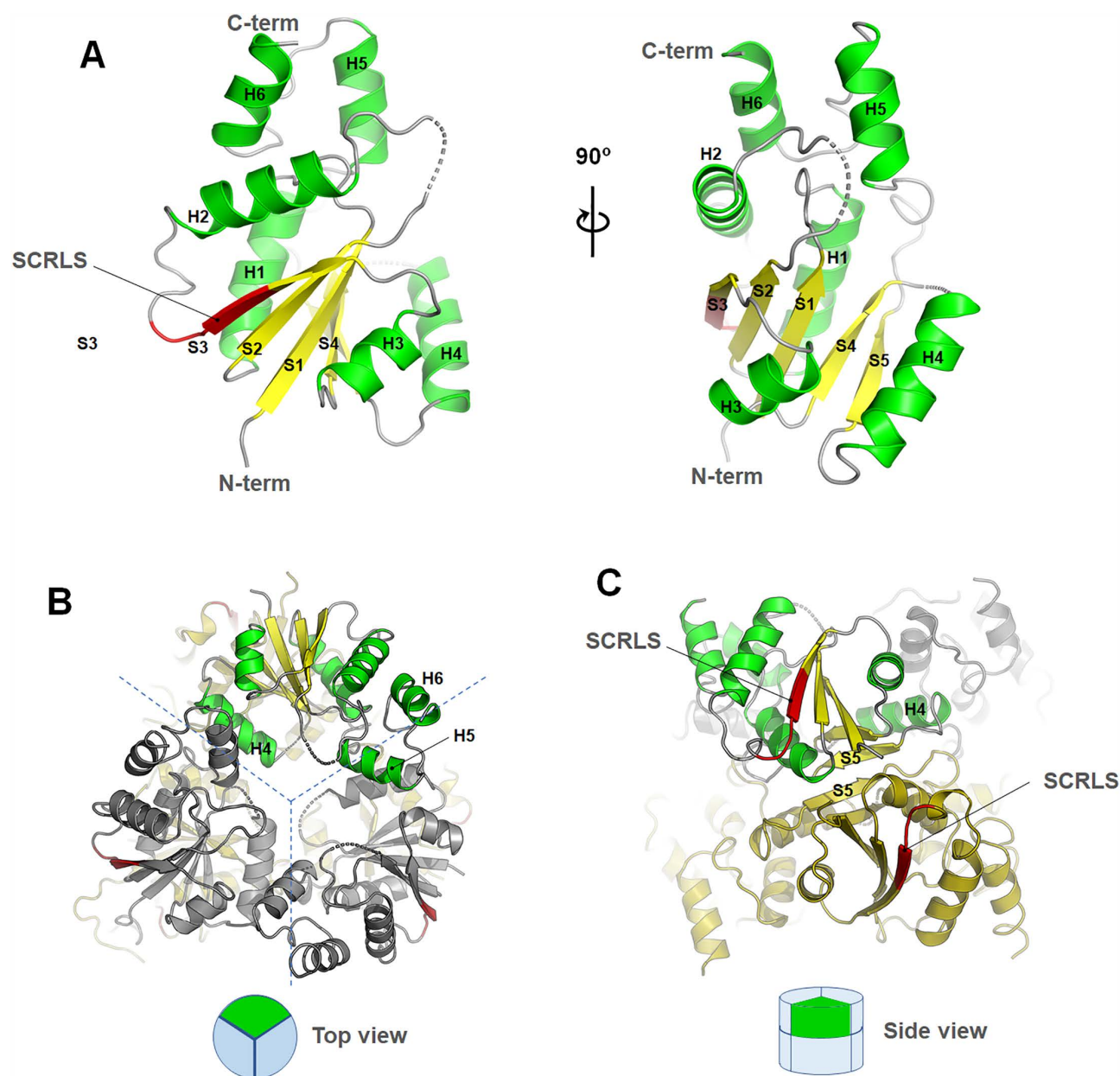


Fig 3. Overall crystal structure and hexameric assembly of *MpaPPAT*. (A) Ribbon diagram of the *MpaPPAT* monomer, shown in two orientations related by a 90° rotation. α -helices (H1-H6) are colored green and β -strands (S1-S5) are yellow. The unique surface loop insertion (SCRLS, residues 67-71), located between H2 and S3, is highlighted in red. N- and C-termini are labeled. (B) Top view of the 'dimer-of-trimers' hexameric assembly. One monomer is colored as in panel A, while adjacent monomers are shown in gray. (C) Side view of the hexamer, illustrating the position of the SCRLS loop at the solvent-exposed surface and strand S5 at the interface.

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among the subunits within this hexameric assembly. Chain D contains a short 3_{10} -helix between S5 and H5, while others remain as a loop in this region (S1A and S1B Figs). At the trimer-trimer interface, the 3_{10} -helix region and S5 interact with S5 of a neighboring monomer. This connection occurs in an antiparallel fashion, linking the subunits by forming a single, large, twisted β -sheet (Fig 3C).

To validate that this hexameric organization is the physiologically relevant form and not merely an artifact of crystal packing, the oligomeric state of *Mpa*PPAT in solution was investigated by sedimentation velocity analytical ultracentrifugation (SV-AUC). The analysis revealed that *Mpa*PPAT behaves as a single, monodisperse species in solution (S2A Fig). The continuous sedimentation coefficient [c(s)] distribution analysis showed a single major peak (99.9%) with a sedimentation coefficient ($s_{20, w}$) of 5.8 S. This peak corresponds to a calculated molecular weight of 99.8 kDa, which is in excellent agreement with the theoretical calculated mass of the hexamer ($6 \times 17.4 \text{ kDa} = 104.4 \text{ kDa}$). This result confirms that *Mpa*PPAT exists as a stable hexamer in solution, validating the biological relevance of the quaternary structure observed in the crystal.

3.4. The SCRLS insertion forms a unique, exposed surface loop

The multi-sequence alignment (Fig 2A) visually confirms the high degree of conservation within the core secondary structure elements (shaded black and gray) across PPAT homologues. However, the most notable feature, clearly visible in the sequence alignment, is the unique insertion (residues 67–71, SCRLS) in *Mpa*PPAT. As hypothesized from the sequence analysis, our crystal structure confirms that this insertion is not a cryptic or internal segment. Instead, it forms a distinct, solvent-exposed surface strand-loop element located between H2 and S3 (Figs 3 and Fig 4 highlighted in red).

This structural feature directly corresponds to the SCRLS insertion highlighted in the alignment. This insertion is notably absent in mesophilic homologues, such as *E. coli*, *Mycobacterium tuberculosis*, and *Bacillus subtilis*. Furthermore, even thermophilic bacteria, including *T. thermophilus* and *T. maritima*, also possess a short turn at the equivalent position (Fig 4). This structural comparison underscores that the elongation of this loop is the key adaptive feature in *Mpa*PPAT.

The presence of such an extended, flexible loop is a common adaptive strategy in cold-active enzymes, as it can enhance the conformational flexibility required for substrate binding and catalysis at low temperatures. Therefore, the

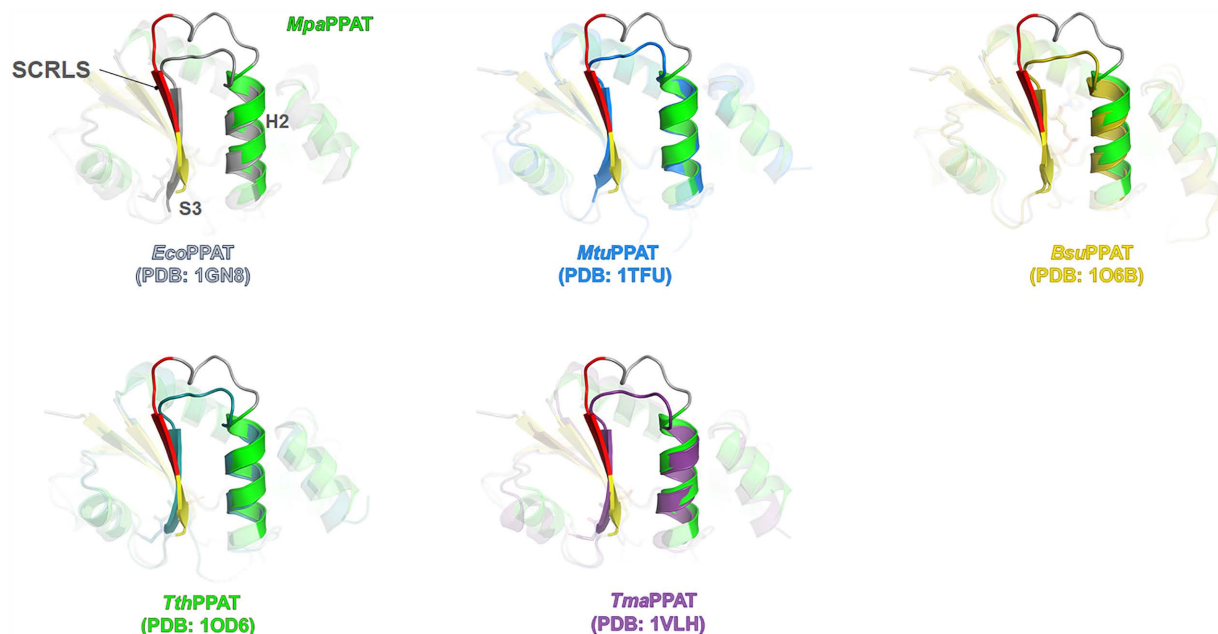


Fig 4. Structural comparison of the SCRLS loop region in *Mpa*PPAT and its homologues. (A) Comparison of the surface loop structure located between α -helix H2 and β -strand S3. The psychrophilic *Mpa*PPAT (top left, superimposed with *Eco*PPAT) features a distinct loop insertion (SCRLS) highlighted in red. This extended loop is notably absent in mesophilic homologues, including *Eco*PPAT (gray), *Mtu*PPAT (blue), and *Bsu*PPAT (gold), as well as in thermophilic homologues *Tth*PPAT (cyan) and *Tma*PPAT (purple). The PDB code for each structure was indicated.

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elongation of this loop may be the key adaptive feature in *MpaPPAT*. To ascertain the functional significance of this unique structural element, we proceeded to generate a loop-deletion mutant (*MpaPPAT*($\Delta 67-71$); p.Ser67_Ser71del) for comparative biochemical and structural characterization.

3.5. The SCRLS loop is critical for low-temperature catalytic activity

To investigate the functional significance of the additional surface loop, we performed comparative steady-state kinetic analyses of wild-type *MpaPPAT* and the loop-deletion mutant *MpaPPAT*($\Delta 67-71$). The catalytic activity was measured using a reverse-direction, two-stage coupled assay (Fig 5A). In the first stage, the adenylyltransferase reaction (generating ATP from dPCoA and PPi) was performed with either wild-type *MpaPPAT* or *MpaPPAT*($\Delta 67-71$) at various incubation temperatures (ranging from 10 °C to 60 °C) for 1 min. In the second stage, the amount of ATP produced in the first

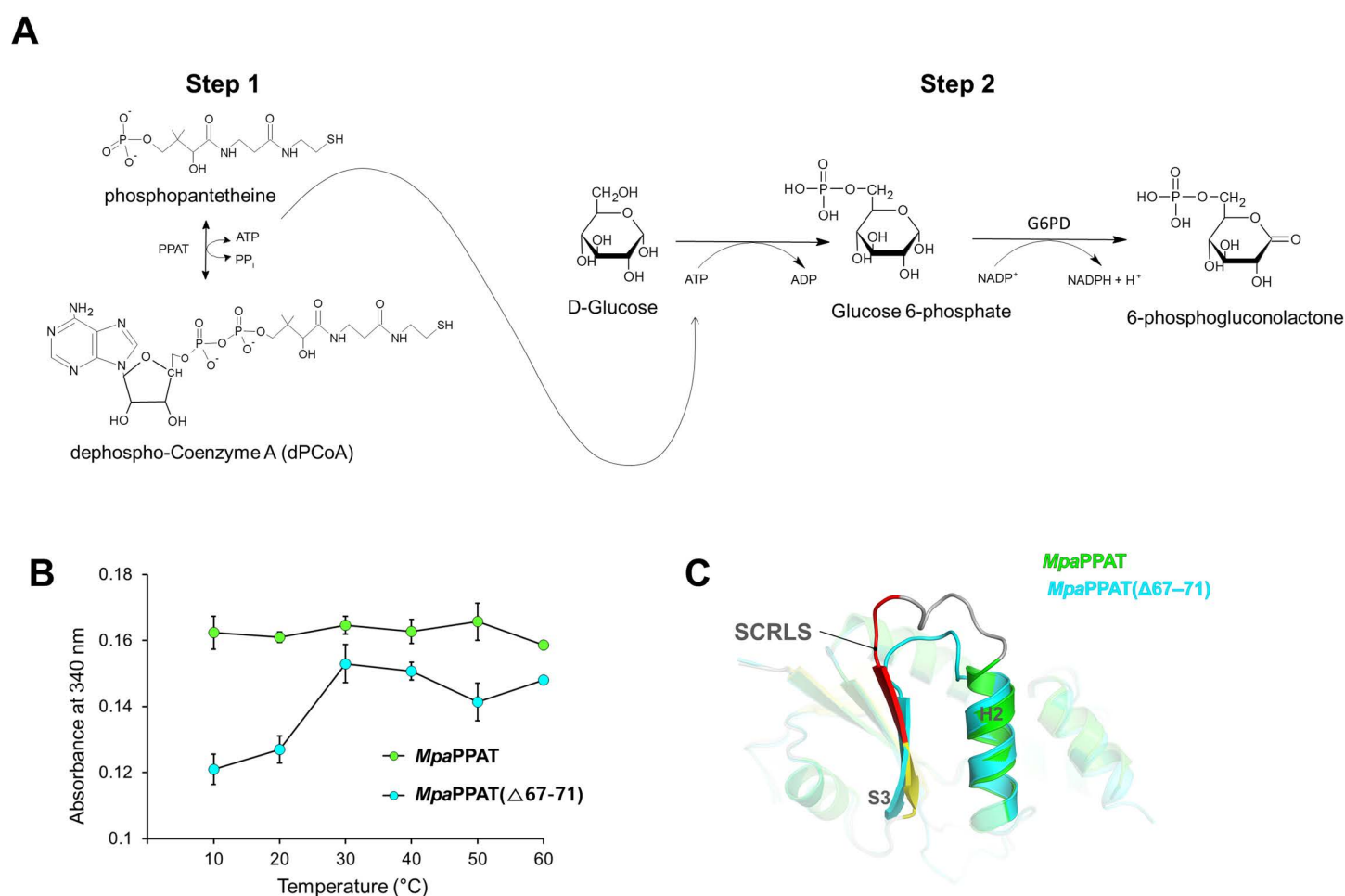


Fig 5. Functional and structural effects of the SCRLS loop deletion. (A) Schematic representation of the two-step coupled assay used to quantify PPAT catalytic activity. (B) Comparative analysis of temperature-dependent activity between wild-type *MpaPPAT* (green) and the *MpaPPAT*($\Delta 67-71$) mutant (cyan). The wild-type enzyme sustains high catalytic activity across a broad low-temperature range (10–30 °C), whereas the loop-deletion mutant shows markedly reduced activity, especially at 10 °C and 20 °C. Activity was quantified by monitoring NADPH absorbance at 340 nm in a two-stage coupled assay. Data are presented as mean S.D. (n=3). (C) Structural superposition illustrating the effect of the loop deletion. The wild-type *MpaPPAT* (green) features the distinct, solvent-exposed loop element. In the *MpaPPAT*($\Delta 67-71$) mutant (cyan), this entire loop is deleted, resulting in a short, truncated turn, as expected.

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reaction was quantified. This was achieved by using the ATP as a substrate for a coupling system comprising hexokinase and glucose-6-phosphate dehydrogenase (G6PD), which stoichiometrically converts NADP⁺ to NADPH. The total ATP generated by PPAT was thus indirectly quantified by measuring the end-point absorbance of NADPH at 340 nm.

Enzymatic activity is generally highest at an optimal temperature (often 30–40 °C for mesophilic enzymes) and decreases sharply at lower temperatures due to reduced thermal energy. The *Mpa*PPAT(Δ67–71) mutant generally followed this expected trend (Fig 5B). Its activity was minimal at 10 °C and 20 °C, peaked at 30 °C, and then declined at higher temperatures. In contrast, the wild-type *Mpa*PPAT defied this trend. Instead of losing activity, the WT enzyme maintained high catalytic activity even as the temperature decreased. It exhibited sustained activity (> 80%) across the entire temperature range of 10 °C to 30 °C. To exclude the possibility that the observed difference in activity was caused by the disruption of the quaternary assembly, we analyzed the oligomeric state of the mutant using sedimentation velocity analytical ultracentrifugation (SV-AUC). The sedimentation velocity profile confirmed that the *Mpa*PPAT(Δ67–71) mutant maintains an intact hexameric structure in solution, indistinguishable from that of the wild-type (S2B Fig). This ensures that the functional impairment is attributed to the specific loss of the surface loop rather than a global destabilization of the hexameric complex.

These results demonstrate that the SCRLS surface loop is the key structural feature responsible for maintaining high catalytic performance at low temperatures, thereby defining the enzyme's cold-active properties. Superimposed structures of wild-type *Mpa*PPAT and *Mpa*PPAT(Δ67–71) mutant support this structural interpretation (Fig 5C).

3.6. Structural basis for the impaired activity of *Mpa*PPAT(Δ67–71) mutant

To investigate the structural basis for the impaired low-temperature activity of the *Mpa*PPAT(Δ67–71) mutant, we determined its crystal structure to a comparable resolution (2.56 Å, S2 Table). A detailed structural comparison between the wild-type and the *Mpa*PPAT(Δ67–71) mutant revealed a similar overall structure with an RMSD of 0.662 Å for the hexamer. However, a critical conformational change was observed, not only at the loop deletion site but also deep within the hexameric core interface (Fig 6).

In the wild-type structure, the loop region connected to H4 exhibited poorly defined electron density, indicating a high degree of intrinsic flexibility (S3 Fig). This flexibility appears to be a crucial characteristic of the active enzyme, likely acting as a “flexible gate” for substrate entry and product release. In contrast, the *Mpa*PPAT(Δ67–71) mutant structure showed clear, well-defined electron density and interactions among monomers in this region (Fig 6A, S3 Fig). It is worth noting that this interface represents the internal core of the biological hexamer and is isolated from crystal packing contacts. Therefore, the well-defined electron density observed in the mutant indicates an intrinsic stabilization of the hexameric assembly induced by the mutation, rather than being a result of lattice interactions. The deletion of the remote surface-exposed SCRLS loop appears to induce a long-range conformational change, causing this H4-associated loop to stabilize. This stabilization allows the H4 helices from adjacent subunits to meet, effectively “clamping” the core of the complex.

This conformational change also introduces a shift in the electrostatic properties of the central pore. As shown in the electrostatic surface potential analysis (Fig 6B), the wild-type's flexible central pore is characterized by a significant positive charge. Structural inspection reveals that this positive potential is predominantly generated by a cluster of solvent-exposed basic residues, including H39, K42, R51, R93, R96, and R138. This positively charged channel likely serves as an electrostatic “funnel” to attract and “steer” the negatively charged substrates (ATP and phosphopantetheine) toward the active sites. This “electrostatic steering” mechanism is consistent with structural studies of *Eco*PPAT, which reported that the mouth of the solvent channel is lined with positively charged residues to guide substrates [28]. Conversely, in the *Mpa*PPAT(Δ67–71) mutant, the newly stabilized and clamped core structure exposes negatively charged residues, specifically D92 (D97 in WT) and D95 (D100 in WT), creating an electrostatic barrier that would repel these incoming substrates.

We propose that this dual mechanism of electrostatic steering and dynamic gating is essential for maintaining high catalytic activity at low temperatures. This conclusion is strongly supported by an analysis of other homologues (S4 Fig).

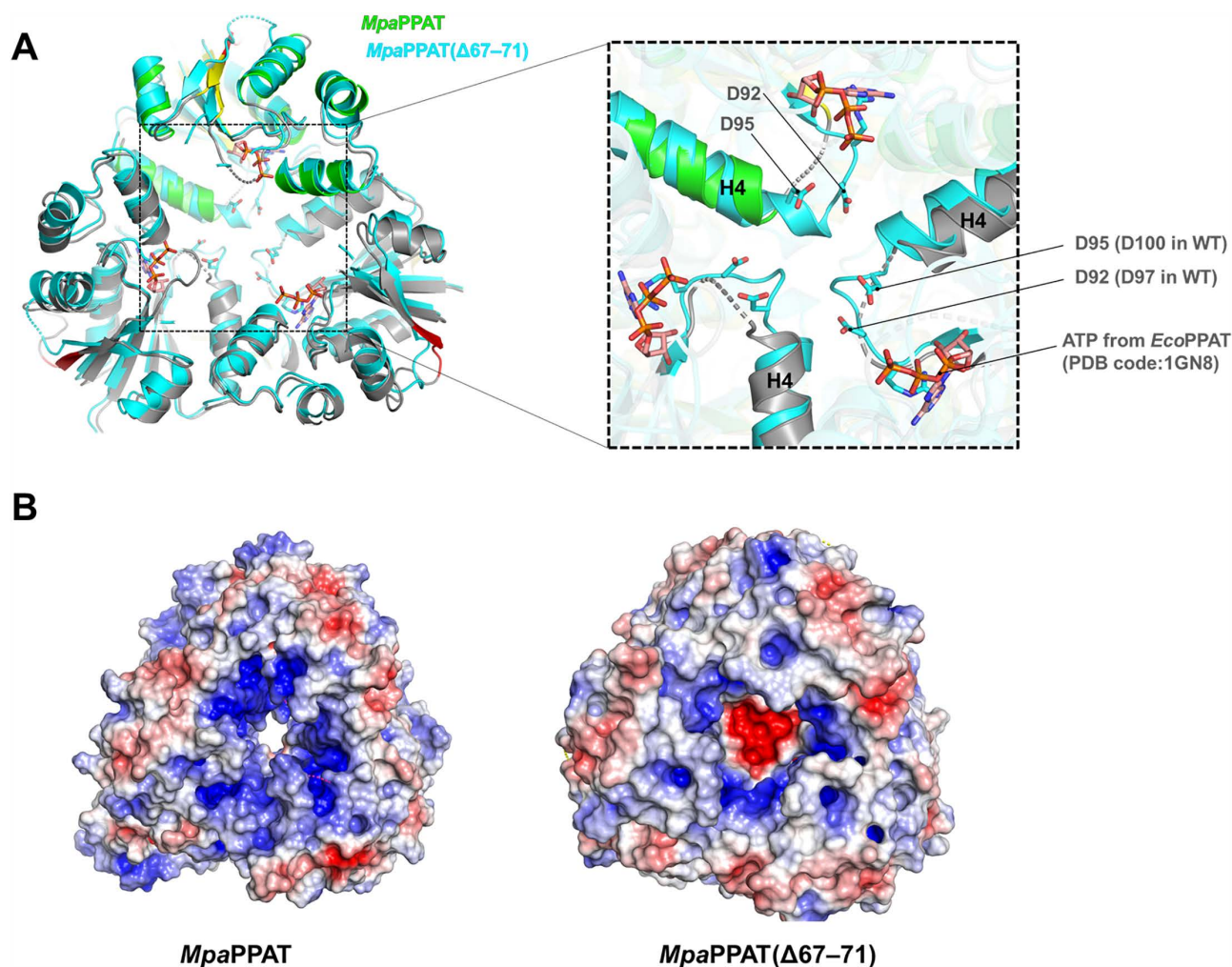


Fig 6. Comparison of structural and electrostatic basis in the *MpaPPAT* and *MpaPPAT(Δ67–71)* mutant. (A) Superposition of the hexameric core structures of wild-type *MpaPPAT* (green) and the *MpaPPAT(Δ67–71)* mutant (cyan). The close-up view highlights the conformational changes at the trimer-trimer interface, particularly around α -helix H4. The deletion of the distal SCRLS loop induces a long-range effect that stabilizes the H4-associated loop in the mutant (involving residues D92 and D95), leading to a “clamped” and rigidified core. An ATP molecule (stick model) from the *EcoPPAT* structure (PDB code 1GN8) is superimposed to illustrate the proximity of the active site to the clamped interface. (B) Electrostatic surface potential comparison of the central pore (viewed along the 3-fold axis). The wild-type enzyme (left) features a wide, positively charged (blue) central channel, essential for the electrostatic steering of negatively charged substrates. Conversely, the mutant (right) displays a constricted, negatively charged (red) pore, creating an electrostatic barrier that repels substrates. Blue and red represent positive and negative potentials, respectively (± 5 kT/e).

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Most non-psychrophilic counterparts (except *BsuPPAT*, where the equivalent acidic residue, D95, is structurally buried inward, resulting in a neutral central pore) display a constricted, negatively charged central funnel, sharing features with the impaired mutant rather than the cold-active wild-type. Therefore, the wild-type’s flexible, positive pore represents a specific evolutionary strategy for cold adaptation.

An allosteric network links the remote SCRLS loop (residues 67–71 in *MpaPPAT*) to the active site. The deletion of this loop transmits a signal that stabilizes the H4 helix. These findings indicate that the loop is not a passive feature but an active allosteric modulator, which functions to maintain the dynamic and flexible state of the hexameric assembly. Although the precise mechanism of this signal transduction remains elusive, the presence of long-range allosteric networks in PPAT

has been previously reported. For example, *P. aeruginosa* PPAT utilizes an “arginine switch” mechanism (residues R90 and D94) to regulate the transition between the enzyme’s catalytic and inhibitory states [36]. This aligns with the findings of this study, which identify the SCRLS loop of *MpaPPAT* as a specific allosteric modulator for cold adaptation.

In summary, our study demonstrates that the insertion of the five-amino-acid surface-exposed loop, a feature associated with psychrophilic organisms, is critical for cold adaptation. However, the precise timescale and atomic details of how this loop influences the core flexibility remain to be fully defined. Future molecular dynamics (MD) simulations will therefore be necessary to elucidate the specific signal transduction pathway and map how the dynamics of this surface loop modulate the global structure and activity of *MpaPPAT*.

4. Conclusion

In this study, the structural and functional basis of cold adaptation for phosphopantetheine adenylyltransferase (PPAT) from the psychrophilic bacterium *Methylocapsa palsarum* was elucidated. A unique surface loop insertion, conserved among psychrophilic homologues, was identified, and its critical role in maintaining high catalytic activity at low temperatures was demonstrated.

The central finding of this work is the discovery of a long-range allosteric network that couples this distant surface loop to the enzyme’s catalytic machinery. Comparative structural analysis of the wild-type and the loop-deletion mutant, *MpaPPAT*(Δ67–71), provides compelling evidence for this connection. The deletion of the surface loop did not cause a simple local disruption but instead triggered a significant conformational reorganization within the hexameric core. This reorganization results in a dual impairment that explains the loss of cold activity. First, it leads to the rigidification of the H4 helix, “clamping” the active site and restricting the essential conformational flexibility (the “flexible gate”) required for efficient catalysis. Second, this clamping induces an electrostatic shift; the unique positively charged central pore of the wild-type, which serves to attract negatively charged substrates, is lost and replaced by a negatively charged pore. Analysis of non-psychrophilic homologues further validates this finding, as they also possess a negatively charged and constricted central pore, similar to the impaired mutant. This suggests that the wild-type’s flexible, positively charged channel is a key evolutionary strategy for cold adaptation, enabling it to overcome the kinetic challenges of low-energy environments.

While these structures provide static snapshots of this allosteric communication, the precise mechanism of signal transduction from the surface loop to the catalytic core remains to be fully elucidated. This work establishes *MpaPPAT* as an excellent model for studying allosteric regulation in complex multimeric enzymes. Future studies, particularly molecular dynamics (MD) simulations, are warranted to map this allosteric pathway in detail and visualize how the dynamics of the surface loop influence the global conformational energy landscape of the hexamer.

In conclusion, this study provides new insight into how enzymes evolve sophisticated adaptive strategies. It demonstrates that the insertion of a simple loop can act as a remote allosteric switch, fine-tuning not only the dynamic properties but also the electrostatic environment of the entire complex to overcome the biophysical challenges of extreme environments.

Supporting information

S1 Table. Plasmids and primers used in this study.

(PDF)

S2 Table. X-ray diffraction data collection and refinement statistics.

(PDF)

S1 Fig. Conformational heterogeneity within the *MpaPPAT* hexamer. (A) Structural comparison of two distinct monomer conformations observed within the *MpaPPAT* hexamer. The loop region of Chain A between strand S5 and helix H5 is flexible, and lacks a defined secondary structure (green). In contrast, the same region in Chain D adopts a short 3_{10} -helix

conformation, highlighting the structural plasticity of this segment. The unique SCRLS loop is colored red in both chains. (B) Close-up view of the trimer-trimer interface. The short 3_{10} -helix region of one monomer interacts with the adjacent monomer (yellow oval), contributing to the stabilization of the hexameric assembly.

(PDF)

S2 Fig. Sedimentation velocity analytical ultracentrifugation (SV-AUC) analysis of *Mpa*PPAT variants. (A) Sedimentation velocity analysis of wild-type *Mpa*PPAT. The top panel displays the sedimentation boundaries fitted to the Lamm equation, with residuals plotted below. The bottom panel shows the molecular weight distribution, revealing a single predominant peak at approximately 99.8 kDa, corresponding to a hexameric oligomer. (B) Sedimentation velocity analysis of the *Mpa*PPAT($\Delta 67-71$) mutant. The data (top) and molecular weight distribution (bottom) demonstrate that the mutant exhibits a major peak at approximately 98.1 kDa. This confirms that the mutant retains a stable hexameric assembly in solution, comparable to that of the wild-type protein.

(PDF)

S3 Fig. Electron density comparison at the hexameric core interface. The $2F_o - F_c$ electron density maps (gray mesh) are contoured at 1.0σ for the central interface region, covering residues Gly89 to Met107 shown as sticks. Distinct subunits are differentiated by color. The core interface residues of wild-type *Mpa*PPAT are widely separated with relatively poor electron density in the loop regions, indicative of the high intrinsic flexibility and open conformation required for the “flexible gate” mechanism. In contrast, the *Mpa*PPAT($\Delta 67-71$) mutant structure displays well-defined electron density and tight packing between adjacent subunits.

(PDF)

S4 Fig. Comparison of electrostatic surface potentials among *Mpa*PPAT and homologues. Surface representation of the hexameric PPAT structures viewed along the 3-fold axis, colored according to electrostatic potential (blue = positive, red = negative, white = neutral; scale $\pm 5kT/e$). Wild-type *Mpa*PPAT displays a wide, positively charged (blue) central pore. In contrast, *Eco*PPAT, *Mtu*PPAT, *Tth*PPAT, and *Tma*PPAT exhibit a negatively charged central region similar to the impaired mutant, whereas *Bsu*PPAT shows a more neutral central pore. This comparison highlights the unique positive electrostatic character of the *Mpa*PPAT central channel as a key feature for cold adaptation.

(PDF)

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