Structural Basis for the ATP-dependent Configuration of Adenylation Active Site in *Bacillus subtilis* o-Succinylbenzoyl-CoA Synthetase*

Yaozong Chen, Yueru Sun, Haigang Song, and Zhihong Guo

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From the Department of Chemistry and State Key Laboratory for Molecular Neuroscience, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China

Background: It is not clear how the adenylation active site is formed in adenylylating enzymes.

**Results:** ATP binding induces adenylylate-forming conformation, creates a binding site for the carboxylate substrate, and aligns active site residues for catalysis.

Conclusion: ATP configures the adenylation active site.

Significance: A new structural role is revealed for ATP in forming the active adenylation conformation in the catalysis of adenylylating enzymes.

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**o-Succinylbenzoyl-CoA synthetase, or MenE, is an essential adenylylate-forming enzyme targeted for development of novel antibiotics in the menaquinone biosynthesis. Using its crystal structures in a ligand-free form or in complex with nucleotides, a conserved pattern is identified in the interaction between ATP and adenylylating enzymes, including acyl/aryl-CoA synthetases, adenylylation domains of nonribosomal peptide synthetases, and luciferases. It involves tight gripping interactions of the phosphate-binding loop (P-loop) with the ATP triphosphate moiety and an open-closed conformational change to form a compact adenylylation active site. In MenE catalysis, this ATP-enzyme interaction creates a new binding site for the carboxylate substrate, allowing revelation of the determinants of substrate specificities and in-line alignment of the two substrates for backside nucleophilic substitution reaction by molecular modeling. In addition, the ATP-enzyme interaction is suggested to play a crucial catalytic role by mutation of the P-loop residues hydrogen-bonded to ATP. Moreover, the ATP-enzyme interaction has also clarified the positioning and catalytic role of a conserved lysine residue in stabilization of the transition state. These findings provide new insights into the adenylylation half-reaction in the domain alteration catalytic mechanism of the adenylylate-forming enzymes.**

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MenE is homologous to acyl/aryl-CoA synthetases (ACS) and catalyzes a similar two-step reaction. In the first adenylation step, the OSB substrate reacts with Mg$^{2+}$-ATP to form the adenylylate intermediate, OSB-AMP, which subsequently reacts with the third substrate, coenzyme A, to form OSB-CoA in the second thioesterification step (Fig. 1). The same adenylation step is found in the reactions catalyzed by the adenylation domains of nonribosomal peptide synthetases (NRPS) and firefly luciferases, which together with acyl/aryl-CoA synthetases form the ANL family of adenylylating enzymes (9). Over the last several decades, several members of this enzyme family, including MenE, have been kinetically analyzed to adopt an ordered Bi Uni Uni Bi ping-pong mechanism, which involves ATP as the first binding substrate and a large conformational change during the catalysis (6–8).

Early structural and related biochemical studies of adenylylating enzymes in the ANL family were focused on luciferases and the adenylation domains of NRPS, which were found to be highly conserved in the three-dimensional structure consisting of a large N-terminal domain (N-domain) containing ~400–550 residues and a smaller C-terminal domain (C-domain) containing ~130 residues (16–18). Ten conserved motifs, namely A1–A10, were identified to play important structural and catalytic roles in the enzymatic reactions, of which the A3 motif is the most conserved among the ANL family (19). This conserved A3 motif overlaps with “motif I,” which defines a consensus
sequence of (T/S)(S/G)(T/S)(T/S/E)GX(P/Q/M/T)K (20) and is homologous to the conserved phosphate-binding loop (P-loop) involved in binding of triphosphate by ATP- and GTP-binding kinases (21). However, the active site formed at the interface of the N- and C-domains in the early crystal structures is only suitable for the first-step formation of the adenylate intermediate from the carboxylate and ATP substrates.

In 2003, Gulick and co-workers (22) used a nonhydrolyzable adenylate, adenosine 5'-propyl phosphate, and coenzyme A to trap the bacterial acetyl-CoA synthetase in a different conformation in which the C-domain is rotated around a hinge residue by 140° to form a new active site suitable for the second-step thioesterification. On this basis, a domain alternation mechanism was proposed for all adenylylating enzymes in the ANL family involving two distinct active conformations, one for adenylate intermediate formation and the other for thioester formation (9). These two conformations form two distinct active sites with the same set of N-domain residues but a different set of residues on two opposite sides of the small C-domain, which rotates a large angle after adenylate formation to achieve conformational change and active site re-configuration.

This domain alternation mechanism is strongly supported by the finding that mutation of residues on a different side of the C-domain specifically affects either the first or the second step of the reaction catalyzed by a number of adenylylating enzymes such as 4-chlorobenzoyle-coenzyme A ligase (4CBL) (15), bacterial acetyl-CoA synthetase (23), luciferase (24), and the stand-alone adenylation domain EntE of the enterobactin synthetase (25). At present, both the adenylate-forming and thioester-forming conformations have been structurally characterized for several ANL family members, including EntE from 

\[
\begin{align*}
\text{OSB} + \text{ATP} & \rightarrow \text{OSB-AMP} + \text{PP}_1 \\
\text{OSB-AMP} + \text{CoA-SH} & \rightarrow \text{OSB-CoA} + \text{AMP} + \text{PP}_1
\end{align*}
\]

FIGURE 1. Reaction catalyzed by OSB-CoA synthetase (MenE). A, MenE-catalyzed two-step reaction. B, ordered Bi Uni Uni Bi ping-pong catalytic mechanism.

Experimental Procedures

Chemicals—The MenE substrate OSB was prepared chemoenzymatically from chorismic acid that was isolated from an engineered Escherichia coli strain KA12 (34). It was synthesized in one pot using recombinant EntC (35), MenC (36, 37), MenD (36, 37), and MenH (38–40) and purified by reverse-phase HPLC. The isolated product was lyophilized and re-dissolved in 10 mM Tris (pH 8.0) buffer for determination of its concentration by the UV absorbance at 250 nm (the extinction coefficient $\varepsilon = 4900 \text{ M}^{-1} \text{ cm}^{-1}$). Other chemicals used in this study, including ATP, AMP, CoA, NaHCO$_3$, isopropyl $\beta$-D-thiogalactopyranoside, polyethylene glycol (PEG), (-)-2-methyl-2,4-pentadiol, buffers, and other salts, were purchased from Sigma.

Expression and Purification of BsMenE and Its Mutants—The menE gene was amplified from the genomic DNA of B. subtilis strain 168 using 5'-ATT TGC GGC CGC ATG CTG and 3'-ATT TGC GGC CGC ATG CTG as primers and was inserted into pET28a between NotI and BamHI sites. The subcloned gene was sequenced by Beijing Genomics Institute (BGI, Shenzhen, China) and found to be identical to the sequence deposited in GenBank™ (accession number NP_390957). The protein with a C-terminal hexahistidine tag was overexpressed in E. coli strain BL21 (DE3) in Luria broth (LB) containing 25 $\mu$g/ml kanamycin for 8 h at 37 °C after induction by 0.2 mM isopropyl $\beta$-D-thiogalactopyranoside and purified to homogeneity by Ni$^{2+}$-chelating column chromatography and size exclusion chromatography (HiPrep 16/60 Sephacryl, GE Healthcare). The purified protein was concentrated, flash-frozen in liquid nitrogen, and stored at $-20 ^\circ$C in 10 mM Tris-HCl (pH 8.0) buffer containing 2 mM 2-mercaptoethanol and 10% glycerol. The protein concentration was determined by Pierce™ Coomassie Plus (Bradford) assay kit (Thermo Scientific).

The expression constructs of BsMenE mutants R382A, R382K, T152A, G154P, T155A/T156A, T155A, T156A, and G157P were generated by QuikChange™ site-directed mutagenesis kit (Stratagene) using the BsMenE-expressing plasmid in pET28a as template. All mutants were sequenced by BGI to confirm that only the desired mutations had been incor-
porated into the menE gene. The expression and purification of these mutants followed the same protocol described above for the wild-type BsMenE.

**Activity Assay and Steady-state Kinetics**—The BsMenE activity was determined by a coupling assay using E. coli MenB (6), which was obtained as an untagged recombinant protein as reported previously (41, 42). In the assay, the BsMenE product OSB-CoA was transformed by excess MenB to 1,4-dihydroxyl-2-naphthoic acid-CoA for measurement at 392 nm (molar extinction coefficient ε = 4000 M⁻¹·cm⁻¹) (43). For the single substrate kinetics, a 140-μl standard assay mixture contained 50 mM Tris·HCl (pH 7.5), 20 mM NaCl, 5 mM MgCl₂, 80 μM MenB, 20 mM NaHCO₃ and a varied concentration of the substrate OSB, ATP, or CoA with the other two substrates fixed at a saturation concentration (OSB at 1.0 mM, ATP and CoA at 2.0 mM). The reactions were initiated by addition of BsMenE to a final concentration of 250 nM after a 3-min incubation. The real-time absorbance increase at 392 nm was measured for at least 1 min, and the reaction rate was calculated from the slope of the initial linear region of the reaction progress curve. All activity assays were carried out in triplicate at 24 °C. Subsequently, the data were fit to Michaelis-Menten Equation 1,

\[ v = \frac{V_{\text{max}}[S]}{K_M + [S]} \]  

(Eq. 1)

where \( v \) is the reaction rate (in μM/min); \( V_{\text{max}} \) is the maximum reaction rate (in μM/min); \([S] \) is the substrate concentration (in μM), and \( K_M \) is the Michaelis–Menten constant (in μM).

**Crystallization**—Crystallization conditions were screened using Index Screen and Crystal Screen I and II from Hampton Research or Wizard™ Classic 3 and 4 from Rigaku. Crystallization was carried out with the hanging drop vapor diffusion method at 16 °C. A rod-like crystal (150 × 20 × 30 μm) of ligand-free BsMenE was obtained after a 10-day growth in a 1:1 mixture of a solution of BsMenE at 10 mg/ml and a reservoir solution at pH 6.15 containing 8.1% (w/v) PEG8000, 0.09 M cacodylate/HCl, 0.144 M calcium acetate, 12.6% glycerol, 0.049 M sodium phosphate monobasic, and 0.091 M dibasic potassium phosphate. A trapezoid-like crystal (200 × 100 × 70 μm) of BsMenE·ATP·Mg²⁺ complex was grown within a week in a 1:1 mixture of a solution of 10 mg/ml BsMenE preincubated with 3 mM ATP and 5 mM MgCl₂ and a reservoir solution at pH 7.7 containing 7.2% (v/v) ethylene glycol, 9.9% (w/v) PEG8000, 0.09 M HEPES, 3.5% (v/v) 2-methyl-2,4-pentanediol, and 0.01 M acetate. Single hexagonal crystals of the BsMenE·AMP complex with the longest dimension up to 50 μm were grown in the 1:1 mixture of a solution of 10 mg/ml BsMenE preincubated with 3 mM AMP and a reservoir solution at pH 7.5 containing 0.10 M HEPES, 10% (w/v) PEG6000, 5% (v/v) (±)-2-methyl-2,4-pentadiol. These crystals were soaked in cryoprotectant containing 20% (v/v) glycerol in the mother liquor and then flash-frozen in liquid nitrogen before data collection.

**Data Collection, Processing, and Structural Determination**—X-ray diffraction data were collected for the obtained BsMenE single crystals at the beamline BL17U of the Shanghai Synchrotron Radiation Facility with an ADSC Quantum 315R charge-coupled device detector. Diffraction images were indexed, integrated, and scaled using HKL2000 and iMOSFLM in the CCP4 suite (44). TRUNCATE of the CCP4 package was used to convert the intensities to structure factors. The ligand-free BsMenE structure was solved by molecular replacement with Phaser (45) in the CCP4 suite using the backbone of the N-domain (residue 1–394) in chain A of S. aureus MenE (PDB code 3IPL) as the search model. The initial electron density map showed that two BsMenE polypeptide chains were correctly located in the asymmetric unit in the space group P12₁,1 for the apo-form. After refinement by PHENIX (47), the C-domain (383–486) of apo-MenE was extended via manual model building using Coot (48) followed by refinement with REFMAC (49) and PHENIX. When the refinement was close to completion, TLS anisotropic refinement (50) was performed for both the N- and C-domains. For the binary complex with AMP and ternary complex with ATP and Mg²⁺, the diffraction datasets were processed similarly, and chain A of the full-length apo-BsMenE was used as the search model for structural determination by molecular replacement. Both structures were determined to have a space group of P4₃ and refined using the same strategy as for the apo-protein. Restraints of ATP and AMP were generated and refined using eLBOW (51). The overall quality of the three structural models was assessed by PDB validation, Procheck (52), and MolProbity (53). Statistics for the data collection and structural refinement are summarized in Table 1.

**Structural Analysis and Sequence Alignment**—BsMenE homologs were identified using a PSI-BLAST search and aligned with ClustalW 2.0 (54). Multiple sequence alignments were displayed with ESPript 3.0 (55). PyMOL version 1.3 was used in the structural analysis and generation of all graphics (56). PISA was used to analyze the protein interfaces (57), and CASTp was used to identify the cavities in the protein structures and to calculate their volume (58).

**Docking Simulation**—All docking simulations were run with AutoDock Vina 1.1.2 using the ATP-BsMenE complex as searching macromolecule (59). The grid maps with 1.000 Å spacing were generated by AutoDock Tools (ADT) with the center set at the coordinates 54.113, 157.401, and 8.502 for the x, y, and z directions, respectively, and the grid box was set at a size of 14, 6, and 6 for the three dimensions. Hydrogen bonds and van der Waals interactions were modeled with a Lennard-Jones parameter of 12–10 and 12–6, respectively. The electrostatic grid maps were calculated using the distance-dependent dielectric permittivity of Mehler and Solmajer (60). The docking simulation was run independently 10 times, and the resulting conformations within a root mean square deviation (r.m.s.d.) of 2.5 Å were clustered and analyzed.

**Results**

**Overall Structure**—The crystal structures of ligand-free, AMP-bound, and ATP-bound BsMenE were determined and refined to a resolution of 1.98, 2.60, and 2.82 Å, respectively (Table 1). The structural models have good overall stereochemistry with most residues in the most favorable or allowed regions of the Ramachandran diagram. Ser-293 is the only Ramachandran outlier in every subunit of all three structures, which also takes the same unfavorable conformation in other BsMenE structures determined previously (61). Curiously, the
corresponding residue of BsMenE Ser-293 is also a Ramachandran outlier in the resolved crystal structures of other adenylate-forming enzymes (22). In the three new BsMenE structures, two substantially different polypeptide chains without the histidine tag and the first two residues at the N terminus are present in the asymmetric units. These two subunits form an interface with extensive hydrogen bonding and ionic interactions that are judged to be biologically relevant by PISA (Fig. 2A) (57). This dimeric assembly in the crystal structures is consistent with the homodimeric quaternary structure determined in solution by size exclusion column chromatography.

Both BsMenE subunits in the ligand-free structure (apo-BsMenE) contain a large N-domain (residues 3–380) and a small C-domain (residues 387–486), whereas only one full-length subunit (chain A) is found in the nucleotide-bound structures. The other subunit contains only the large N-domain with many disordered loops in both ATP-BsMenE and AMP-BsMenE structures. A significantly higher number of residues are disordered in the linker region between the two domains and loops in the small C-domain in the ligand-free subunits than in the full-length nucleotide-bound subunits of the other two structures. Besides the nucleotide ligand, an ethylene glycol molecule from the crystallization buffer is found in a cavity next to ATP in the ATP-BsMenE structure. Interestingly, the C-domain takes a very different orientation relative to the N-domain to form a “closed” conformation in chain A and an “open” conformation in chain B in the ligand-free structure. By comparison, both nucleotide-bound BsMenE structures take a closed conformation similar to chain A of the apo-structure (Fig. 2B).

Open BsMenE Conformations—The open form of the ligand-free BsMenE (chain B of the apo-structure structure, Fig. 2) is a new addition to the several open conformations found in both subunits of another apo-BsMenE structure in a different space group and the sulfate-bound BsMenE structure, which are different in the distance between the small C-domain and the large N-domain (61). Although it is not possible to compare this new open apo-BsMenE structure with the previously determined structures due to unavailability of the latter in the Protein Data Bank, the multiplicity of the open conformations clearly shows the intrinsic property of the enzyme to be present in these structural forms in the absence of a ligand. Noticeably, a similar open conformation is also present for the apo-form of another MenE orthologue from S. aureus subsp. aureus Mü 50 (PDB code 3IPL), demonstrating that the open conformation is not unique to BsMenE but may be a general structural form for all MenE orthologues.

Actually, open conformations similar to those of BsMenE are also present for other adenylate-forming enzymes (16, 34). A common feature of these conformations is that the small C-domain rotates away from the N-domain at a large angle in comparison with the closed adenylate-forming conformation. However, the rotation angle varies in a wide range and is different for each enzyme. The abundance of these open conformational states clearly indicates the high conformational dynamics of the small C-domain and strongly suggests that it is an intrinsic property of the adenylate-forming enzymes to populate various open conformations with a variable distance between its two domains when substrates or other ligands are absent. In their resting state, these enzymes may also populate closed conformations as suggested by the closed conformations observed in the absence of a ligand for MenE (chain A of apo-BsMenE, Fig. 2B) and other adenylate-forming enzymes such as DhbE (18), 4CBL (29), and DltA (62). These resting conformations form the structural basis for a potential substrate recognition mechanism in which the active closed adenylate-forming conformation is either induced or stabilized from the resting open and closed conformations by binding of the carboxylate or ATP in the first-step adenylation reaction.

### TABLE 1
Data collection and refinement statistics

|                | apo-BsMenE | ATP-BsMenE | AMP-BsMenE |
|----------------|------------|------------|------------|
| PDB codes      | 5BUQ       | 5BUR       | 5BUS       |
| Data collection |            |            |            |
| Space Group    | P12,1      | P41        | P41        |
| Unit cell dimensions |          |            |            |
| a, b, c (Å)    | 82.8, 76.9, 93.9 | 121.8, 121.8, 97.8 | 121.8, 121.8, 98.0 |
| α, β, γ (°)    | 90.0, 114.9, 90.0 | 90.90, 90.90 | 90.90, 90.90 |
| Redundancy (%) | 2.3        | 6.2        | 3.1        |
| Completeness (%) | 98.83 (99.15) | 99.74 (100.00) | 99.46 (99.34) |
| Reflections (unique) | 141,919 (73,620) | 606,644 (49,305) | 2,042,714 (43,794) |
| Rwork | 0.1605/0.1997 | 0.2160/0.2658 | 0.1904/0.2335 |
| Rfree | 0.16/0.1977 | 0.2160/0.2658 | 0.1904/0.2335 |
| Rwork/Rfree | 0.1605/0.1997 | 0.2160/0.2658 | 0.1904/0.2335 |
| Bond length (Å) | 0.008 | 0.011 | 0.009 |
| Bond angle (degree) | 1.03 | 1.35 | 1.21 |
| Ramachandran statisticsa | 97.00/2.78/0.22% | 93.00/6.64/0.36% | 95.00/4.53/0.47% |

*Ramachandran statistics indicate the fraction of residues in the most favored, allowed, and disallowed regions of the Ramachandran diagram.*

Values for the highest resolution shell are given in parentheses.
Closed BsMenE Structures—All three closed BsMenE subunits in the solved crystal structures are closely similar to the adenylate-forming conformations identified for a number of ACS, adenylation domains of NRPS, and firefly luciferases with their interdomain cavities corresponding to the identified active sites of the adenylation step. However, they exhibit a significant difference in the exact orientation of the C-domain (Fig. 2B), which rotates from the large N-domain in a slightly different angle around the linker region between the two domains. This difference is more pronounced between the ligand-free BsMenE and the nucleotide-bound structures with a calculated interdomain cavity of 6880 Å³ for the former and 6521 Å³ for the AMP-bound complex using CASTp (58), although it is much smaller between the two nucleotide-bound structures with the smallest interdomain cavity formed in the ATP-BsMenE structure. Notably, there is another closed BsMenE subunit in the previously determined crystal structure in complex with the OSB-AMP intermediate (61), which is not available for comparison. From the fact that the small C-domain takes two very different orientations in this OSB-AMP-bound BsMenE structure as in the ligand-free BsMenE structure, it is likely that the interdomain cavity in the OSB-AMP-bound closed conformation is similar to that in the ligand-free AMP-bound BsMenE structure and larger than that in the ATP-BsMenE structure.

The most compact ATP-BsMenE structure contains a ligand-binding tunnel with both ends extending to the protein surface (Fig. 2C), which consists of two segments crossed at an almost perpendicular angle that binds the nucleoside and triphosphate moieties of the ATP ligand, respectively. In comparison, the binding tunnel is leaky in the AMP-BsMenE structure with an additional opening to the bulk solvent, while it is hardly formed in the ligand-free closed conformation due to the different C-domain orientation. The compact ATP-binding site is a strong indicator that the adenylation active site is properly formed only in the ATP-BsMenE structure, demonstrating the crucial role of the substrate in configuring the active site. In contrast, the closed apo-BsMenE and AMP-BsMenE structures are merely precursor conformations of this active conformation. In the ATP-bound structure, the buffer molecule ethylene glycol occupies a new pocket formed after ATP binding, which should be the binding site of the second substrate, OSB.

Nucleotide-binding Site—The nucleotide-binding site of BsMenE is composed of many conserved amino acid residues mostly from the large N-domain, including the highly conserved nine-residue P-loop (152TSGTTGKP160) in the A3 motif (amino acids 149–160) (Fig. 3). As shown in Fig. 4A, the adenine ring of the ligand in the ATP-BsMenE structure makes direct hydrophobic contact with the side chain benzene ring of the conserved Tyr-286 in the A5 motif oriented at an almost perpendicular angle on one side and the backbone of the tripeptide Gly-263–Gly-264–Gly-265 on the opposite side, although its 6-amino group forms a hydrogen bond with the backbone carbonyl group of Ser-285. The ATP ribose takes a 3’-endo pucker envelope and is stabilized by bifurcated hydrogen bonds between the side chain carboxylate of the invariant Asp-367 in the A7 motif (amino acids 365–367) and its 2’- and 3’-hydroxyl groups. The α-phosphate of ATP is bound by

**FIGURE 2.** Crystal structure of BsMenE. A, dimeric assembly of ligand-free BsMenE in its asymmetric unit. Chain A (green) is in a closed conformation, and chain B (brown) is in an open conformation. B, superposition of different BsMenE conformations. The open (brown) and closed (green) forms of ligand-free BsMenE are aligned with AMP-BsMenE (cyan) and ATP-BsMenE (gray) according to their N-domains (in Cα lines) to demonstrate the different orientation of the C-domains (schematic). ATP of the ATP-BsMenE structure is presented as sticks to indicate the position of the interdomain active site. C, ATP bound to a tunnel with two ends open to the bulk solvent in the ATP-BsMenE structure. The complex is represented in surface and colored according to domains and conserved motifs as labeled. The arrow denotes a probable exit route for the pyrophosphate product after the adenylation reaction. ATP is shown as sticks with the coordinated Mg²⁺ ion denoted as a green sphere.
**ATP Configures Adenylate-forming Conformation**

### ANL superfamily

| Protein                | P-loop (A3) | Hinge loop (A8) | A10       | A4        | LLG motif |
|------------------------|-------------|-----------------|-----------|-----------|-----------|
| bsMenE_5BQ              | 148 TLYGTESTC  | 379 VLDREDLIIGE  | 469 SINDLR | 191 ALPLPHSGL | 260 LLGCG  |
| ACSM2A_3B7W             | 217 LITYESSTC  | 458 FNGQDIDNNSE  | 555 TKGLQR | 260 IDTGNIML | 334 TVGCGS  |
| CBL_15TH                | 157 FLHYESSTC  | 397 ILGVDMDISE  | 490 KAIVLR | 202 LMLPVYVGG | 277 VFFACAT |
| DkbE_1MDF               | 186 FLIKYESSTC  | 425 VADQDINHRE  | 517 NVKGLR | 229 ALPHHRYPF | 303 LGKSLR  |
| DitaA_9FCC              | 394 VIKYSESSTC  | 490 NVKGRQ       | 490 NVKGRQ | 191 CARPSFCGSL | 266 PLTFCG  |
| Luciferase_LLCI         | 149 LITYESSTC  | 434 IVDLKSXSTYK  | 527 TGLCQR | 240 VVFPKHGGFM | 312 TASGCP  |
| PheA_1amu               | 170 IVYYESSTC  | 409 LYGQDNQKSRP  | 499 NQKLDR | 213 LADBIDASV | 282 LITAGS  |
| LC-FACS_1ULT            | 180 IVYYESSTC  | 430 INGDLDLNFGS  | 522 AQKLDL | 225 VVFPKHGNAM | 299 LUVGAA  |
| ACS_1IR2                | 316 FLFYESSTC  | 571 ILOGDIDVWS  | 673 SDCRR  | 360 AADIGGWTG | 439 LGSGEP  |

### MenE orthologs

| Protein          | P-loop (A3) | Hinge loop (A8) | A10       | A4        | LLG motif |
|------------------|-------------|-----------------|-----------|-----------|-----------|
| B. subtilis      | 148 TLYGTESTC  | 379 VLDREDLIIGE  | 469 SINDLR | 191 APPBPSTI | 260 LLGCG  |
| B. anthracis     | 143 TLYGTESTC  | 379 VLDREDLIIGE  | 469 SINDLR | 186 CMKPVW | 257 LLGCG  |
| S. aureus        | 165 SINTYESSTC  | 391 IVDLKDMDPP  | 481 TKGLQR | 208 VVFPKHGGF | 277 LLGCG  |
| E. coli          | 136 SINTYESSTC  | 347 IVDLKDMDPP  | 435 GDIKR  | 181 VVFPKHGM | 242 LLGCG  |
| L. innocua       | 142 SINTYESSTC  | 365 LYGQDNQKSR  | 499 NQKLDR | 185 ADGKSTT | 255 LLGCG  |
| L. lactis        | 134 SINTYESSTC  | 353 LYGQDNQKSR  | 499 NQKLDR | 177 ILMVPFSG | 244 LLGCG  |
| L. monocytogenes | 140 SINTYESSTC  | 363 LYGQDNQKSR  | 499 NQKLDR | 183 ILMVPFSG | 253 LLGCG  |
| M. morganii      | 139 SINTYESSTC  | 351 IVDLKDMDPP  | 444 GDIKR  | 182 VVFPKHGM | 242 LLGCG  |
| S. flexneri      | 138 SINTYESSTC  | 347 IVDLKDMDPP  | 435 GDIKR  | 181 ILMVPFSG | 243 LLGCG  |

**FIGURE 3. Alignment of sequences associated with substrate binding, structural changes, and transition state stabilization.** Representative members with their PDB identification numbers in the ANL family and representative MenE orthologues are aligned separately with ClustalW 2.0, and only the relevant motifs are presented using ESPript 3.0. The BsMenE_5BQ in the upper alignment is identical to the MenE orthologue from B. subtilis in the lower alignment.

hydrogen bonds with side chains of Thr-289 and Ser-153 at the N terminus of the P-loop, and likely also Lys-471 of the A10 motif (amino acids 465–474) from the C-domain with its δ-NH₃⁺ at a distance of 3.6 and 3.8 Å to one of the acidic oxygen atoms and the bridging oxygen between α- and β-phosphates, respectively. Interestingly, as shown in Fig. 4B, the β₃-γ-phosphate of the ATP ligand is fixed to the position by a large number of polar interactions, including hydrogen-bonding interactions with five consecutive residues (152TSGTT156) of the P-loop, a salt bridge to the γ-phosphate with the invariant Lys-160 at the P-loop C terminus, direct chelating interactions of β₂- and γ-phosphates with the divalent Mg²⁺ ion anchored by the side chain carboxylate of the strictly conserved Glu-290 through mediation of two water molecules, and bidentate hydrogen bonds to the β-phosphate with the positive side chain carboxylate of the strictly conserved Glu-290 in ATP- and GTP-dependent kinases and are likely necessary to complete active site closure. This charged residue likely exerts this structural effect by affecting the backbone torsional angles of the downstream hinge residue Ser-384 to move the C-domain closer to the N-domain. Consistent with this proposed role, this arginine residue is strictly conserved among all ANL family members (Fig. 3).

**Comparison of ATP-bound Adenylate-forming Enzymes—**Several crystal structures with bound ATP or its nonhydrolyzable analogues have been determined for human medium chain acyl-CoA synthase (ACSM2A) (30), d-alanine carrier protein ligase DltA from Bacillus cereus (32), and long chain fatty acyl-CoA synthetase (LC-FACS) from Thermus thermophilus (33). The LC-FACS structure is unique in taking a thioester-forming conformation with the ligand bound to a site far from the P-loop, whereas all other structures are present in the adenylate-forming conformation with the ligand bound close to the P-loop. Among these structures, the ATP-bound BsMenE is most similar to ACSM2A bound to ATP or AMPCP with a residue in shorter chain ACS, and has been proposed to be involved in both the adenylation and thioesterification reactions. In the OSB-AMP-bound BsMenE structure, this histidine residue takes another slightly different rotamer conformation by forming a hydrogen bond with the oxygen atom of the carboxyl group linked to the phosphate group (61), suggesting that it may be involved in binding of the carboxylate substrate, OSB, and stabilizing the transition state of the adenylate reaction.

Besides the different P-loop orientation and the associated different interactions, AMP-BsMenE is different from the ATP-bound structure in the absence of the ligand-mediated domain interaction involving Arg-382, which is apparently correlated to its incomplete closure of the interdomain active site cavity. In the ATP-BsMenE structure, the Arg-382 side chain strongly interacts with the ATP β-phosphate group via the bidentate hydrogen bonds with strong attractive electrostatic interaction and thus likely provides the driving force for the complete active site closure. This charged residue likely exerts this structural effect by affecting the backbone torsional angles of the downstream hinge residue Ser-384 to move the C-domain closer to the N-domain. Consistent with this proposed role, this arginine residue is strictly conserved among all ANL family members (Fig. 3).
similar C-domain orientation relative to the N-domain, despite a limited 19.6% sequence identity between the two proteins. In the ATP-ACSM2A structure (PDB code 3C5E) (30), most of the ligand-enzyme interactions are very similar to those in the ATP-BsMenE structure and involve the same number and the same type of interactions to the same atoms of the ligand (Fig. 5). The only obvious difference is that the invariant A10 Lys-577 forms a hydrogen bond with the β-phosphate in ACSM2A rather than a hydrogen bond to the α-phosphate of ATP by the corresponding A10 residue in BsMenE.

Both the ATP-bound BsMenE and ACSM2A are significantly different from the ATP-DltA structure in the C-domain orientation by as much as ∼48°, although they are all in the adenylate-forming conformation (32). Their nucleotide-binding sites also exhibit significant differences, although they share similar ligand-enzyme interactions for the adenine and ribose moieties.
ATP Configures Adenylate-forming Conformation

FIGURE 5. Structural comparison of BsMenE and ACSM2A in ATP binding. The binding site residues from BsMenE (in yellow sticks) are labeled, whereas their counterparts in ACSM2A (in gray sticks) are presented without numbering. Hydrogen bonding interactions involving the catalytic A10 lysine (Lys-471 in BsMenE) and the A8 arginine (Arg-382 in BsMenE) are denoted by yellow broken lines.

and for the invariant A8 arginine (Arg-397 in DltA) and the Mg$^{2+}$-chelating aspartate (Asp-298 in DltA). Specifically, there are few hydrogen bonds between the triphosphate and the P-loop in the ATP-DltA structure, in contrast to the tight wrapping of the triphosphate by the P-loop in BsMenE and ACSM2A. Actually, the P-loop is located at a distance from the triphosphate in DltA and a large part of it is disordered without contact with ATP. The reason for these different ATP interactions in DltA is unclear, and adverse crystal packing interactions, as implicated previously (32), are one of the possible explanations.

**Mutation of Residues in the P-loop**—The P-loop is highly conserved among all members of the ANL family with a consensus sequence of (T/S)(S/G)G(T/S)(T/S/E)G (residues Thr-152 to Lys-160 in BsMenE) (20). Previous functional characterization of the conserved residues mainly focused on the penultimate proline residue and the strictly conserved glycine and lysine residues. It was found that introduction of hydrophobic isoleucine or negatively charged aspartate to the positions of glycine residues causes more than a 4 orders of magnitude decrease in catalytic efficiency in either 4CBL from *Pseudomonas* sp. strain CBS3 (20) or adenylation domains in NRPS (19). In contrast, changes at the invariant lysine to methionine or other polar residues are tolerated with a moderate decrease in catalytic activity (20, 63, 64), whereas mutations at the proline residue cause enzyme-dependent effects that range from no significant activity change for a proline-to-valine change in an NRPS adenylation domain (65) to complete inactivation for a proline-to-alanine mutation in 4CBL (20). From the ATP-enzyme interactions in BsMenE and ACSM2A (30), it is easy to understand that the two glycine residues in the P-loop have to be conserved to avoid steric repulsion with the bound ATP, whereas the proline and lysine residues are important in forming the $\beta$-turn in the P-loop hairpin to tightly wrap around the triphosphate of the ligand, for which the former directly facilitates the directional change of the polypeptide chain, and the latter stabilizes the turn by forming hydrogen-bonding interactions with the ligand as well as other P-loop residues. To better understand the catalytic contribution of the intense enzyme-ATP interaction (Fig. 4, A and B), the conserved serine/threonine residues in the P-loop interacting with the $\beta,\gamma$-pyrophosphate of the substrate were mutated into alanine individually or in combination. For comparison, the invariant A8 Arg-382 hydrogen-bonded to the $\beta$-phosphate was mutated to alanine or lysine. In addition, proline was also introduced into the positions of the conserved glycine residues. The resulting mutants, namely T152A, T155A, T156A, T155A/T156A, R382A, R382K, G154P, and G157P, were successfully obtained in high purity with the same stability and circular dichroism spectra as the wild-type enzyme (data not shown), indicating the absence of major structural change caused by the mutations.

The single-substrate kinetic constants $k_{cat}$, $K_{M}$, and $k_{cat}/K_{M}$ of the mutants and the wild-type enzyme are listed in Table 2. It is interesting to note that the kinetic constants are moderately affected for the proline mutants of the conserved glycine residues, leading to a 20–95-fold decrease for ATP and less than a 15-fold decrease for other substrates in $k_{cat}/K_{M}$ in comparison with the wild-type enzyme. These moderate changes indicate that the introduced proline causes a much smaller steric repulsion in comparison with isoleucine in 4CBL (20), and it has only a mild effect on the P-loop backbone orientation, which likely depends more on the multiple hydrogen bonds between ATP and the P-loop residues. In contrast, the threonine-to-alanine mutations cause a much larger decrease in catalytic activity. The resulting mutants exhibit a general $k_{cat}$ decrease of about 2 orders of magnitude for all substrates and a significant substrate-dependent $K_{M}$ increase, which is 100–200-fold for ATP, 2–7-fold for OSB, and 1–6.8-fold for CoA-SH. Overall, the decrease in the catalytic efficiency ($k_{cat}/K_{M}$) is 4800–24,000-fold for ATP, 180–1700-fold for OSB, and 62–190-fold for CoA-SH in single mutants and significantly higher for the double mutant T155A/T156A with a 81,000-fold decrease for ATP and a 1700-fold decrease for the other two substrates. In comparison, the R382A and R382K mutations cause similar $k_{cat}$ and $k_{cat}/K_{M}$ decreases for both ATP and OSB with an extent comparable with that of the T156A mutant (Table 2). However, these two mutants cause a higher $K_{M}$ increase for the coenzyme A substrate than T156A, suggesting that Arg-382 plays a critical role in both the adenylation and the thioesterification step. In contrast, all the P-loop mutants mainly affect the first-step reaction.

It is interesting to note that $k_{cat}$ is significantly decreased by about 2 orders of magnitude when one or two hydrogen bonds are removed by the mutations from the numerous binding interactions for the $\beta,\gamma$-pyrophosphate of the ATP substrate.
leaving bilizing the transition state through their interactions with the reaction in the catalytic process. These residues may con-tribute (Fig. 4) with other active site residues and the metal cofactor may also col. As shown in Fig. 6, the final model was chosen from five with bound between a loop containing the tripeptide261LLG263 with (Fig. 4), demonstrating that the conserved residues not only are involved in ATP binding but also affect subsequent adenylation reaction in the catalytic process. These residues may contribute to the chemical reaction by keeping the ATP substrate in optimal position and orientation for the reaction and by stabilizing the transition state through their interactions with the leaving β-phosphate group. In addition, these residues together with other active site residues and the metal cofactor may also be involved in activating the scissile phosphorus–oxygen bond by straining it via the observed tight binding of the ATP sub-strate (Fig. 4A).

Modeling of the OSB Substrate into Its Binding Site—The OSB substrate was successfully modeled by AutoDock Vina into the binding site adjacent to the bound ATP in its complex with BsMenE after removing the buffer molecule, ethylene glycol. As shown in Fig. 6, the final model was chosen from five structures resulting from the docking simulations on the dataset of its higher similarity to the OSB-AMP-bound BsMenE structure (61) in the interactions between the OSB moiety and the enzyme. As in the adenylate-bound BsMenE structure, OSB is bound between a loop containing the tripeptide261LLG263 with the hydrophobic leucine side chains interacting with the benzene ring of the ligand and the loop containing the A4 motif (196H145). All these binding site residues are specifically conserved among the MenE family members but not among different adenylation enzymes (Fig. 3). Other residues lining the substrate-binding site include Ser-293, Gln-294, Ser-237, Gly-287, Lys-471, and the bound ATP.

Despite their similarities, the docked structure and the adenylate-bound structure exhibit marked differences. Nota-bly, OSB in the docked structure takes a conformation with its benzene ring twisted at an angle to both the 2-carboxy and the carbonyl function of the succinyl group, whereas the OSB moiety in the adenylate-bound structure takes a different conformation with its benzene ring perpendicular to the 2-carboxy and coplanar with the carbonyl function of the succinyl group. In addition, the conformation of the ethylene moiety of the ligand is gauche in the former and anti in the latter. Other differences include the absence of hydrogen bonding of the His-196 side chain imidazole and the Ser-293 side chain hydroxyl to the ligand in the modeled structure, which is present in the adenylate-bound structure due to rotameric flipping of the side chain functional groups and is believed to occur in the binding of OSB by the ATP-bound BsMenE. In the modeled complex, one oxygen atom of the succinyl carboxylate is close to the α-phosphate of ATP with a short oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows oxygen-to-phosphorus distance of 3.5 Å and is opposite the pyrophosphate group of ATP. This positioning of OSB allows.
as MenE (6), 4CBL (15), and ACS with various chain length specificities (11–14). Binding of ATP is the critical first step in the complex catalytic mechanism of these enzymes but is not fully understood. Reported structures of three adenylating enzymes complexed with ATP or its analogues have presented three different pictures for the important ATP-enzyme interaction. In this work, we have successfully determined the structure of ATP-bound BsMenE with its P-loop tightly wrapping around the triphosphate as expected for the same structural motifs in ATP- or GTP-dependent kinases (21). This enzyme is found to be highly similar to human ACSM2A in binding ATP with all but one of the numerous ligand-enzyme interactions conserved between the evolutionarily distal proteins. Remarkably, all the amino acid residues involved in the ATP-enzyme interaction are highly or even strictly conserved, suggesting an identical ATP-binding mode and the same functional and structural effects for ATP binding in all ANL family members. Thus, together with the previous findings for the interaction of ATP with human ACSM2A (30), this ATP-BsMenE complex structure provides a highly conserved structural model for ATP interaction with ANL enzymes to form the active adenylate-forming conformation. Currently, it is not clear why the nucleotide substrate is bound to a site far from the P-loop in LC-FACS (33).

An important component of this conserved ATP-enzyme interaction is the ligand-dependent open-closed conformational change that emerges from current and numerous previous studies. For BsMenE, co-existence of both open and closed conformations in the ligand-free enzyme (Fig. 2), together with the open structures previously determined (61), is a clear indication that the enzyme populates both the open and closed conformations in its resting state. Considering the tight gripping interactions for the ATP triphosphate that makes a 90° turn in the binding tunnel, the compact ATP-BsMenE complex is much more likely to form from an open conformation rather than a closed conformation similar to the active adenylate-forming conformation. On the basis of the fact that open conformations similar to those of BsMenE have been found in the absence of a ligand for other adenylating enzymes in the ANL family (16, 34), such an open-closed conformational change is proposed to generally occur as the first step to form the adenylate-forming active site in catalysis of all ANL enzymes. Actually, a similar open-closed conformational change has been proposed for the formation of the adenylation active site in catalysis of DltA, although no open conformations have been found for the ligand-free enzyme (26).

The slight C-domain orientation difference between AMP- and ATP-bound complexes (Fig. 2B) indicates that there exist structural elements safeguarding the formation of the compact adenylation active site in the open-closed conformational change. Previously, the strictly conserved A10 lysine residue was proposed to play such a role on the basis of its hydrogen bonding to the ATP β-phosphate in ACSM2A (30). However, the A10 Lys-471 in BsMenE makes the same hydrogen-bonding contact with the α-phosphate of the ligands as in other adenylating enzymes (17, 18) and is unable to account for the slight structural difference between the AMP-bound and ATP-bound complexes. In contrast, the invariant A8 Arg-382 forms bidentate hydrogen bonding with the ATP β-phosphate in the AMP-BsMenE complex but makes no contact with the ligand in the AMP-BsMenE complex. This is the only residue that makes differential interactions in the two complexes, which may impact the C-domain orientation by affecting the torsional angles of the downstream hinge residue (Ser-384 in BsMenE) in the linker region between the N- and C-domains. This invariant A8 residue is therefore believed to be crucial for proper formation of the adenylation active site, which is consistent with its pronounced effect on the enzyme activity (Table 2). Other residues at the domain interface are involved in the same interactions in the two nucleotide-bound complexes.

Another important part of the ATP configuration of the adenylation active site is to create a new binding site for the carboxylate substrate. This new binding pocket is successfully defined in BsMenE by molecular docking with the OSB sub-
strate and comparison with the OSB-AMP-bound BsMenE structure (Fig. 6), which is composed of amino acid residues specifically conserved among the MenE family (Fig. 3). Importantly, the two substrates are perfectly positioned for in-line backside nucleophilic substitution reaction with a short oxygen-to-phosphorus distance between the reacting functional groups (Fig. 6). This allows us a glimpse of how the enzyme sequentially binds the two substrates and aligns them just before the reaction happens. It also provides a general model for alignment of the two substrates for the first half-adenylation reaction catalyzed by other ANL enzymes.

In addition to creating a new substrate binding, the ATP binding also positions conserved amino acid residues for stabilization of the transition state. Noticeably, the ATP-bound BsMenE shows for the first time hydrogen bonding interaction of the invariant A10 Lys-471 with the alpha-phosphoryl group of the ATP substrate, strongly supporting the proposed stabilization of the developing negative charge in forming the transition state of the first step adenylation (32). The fact that this strictly conserved lysine interacts with the beta-phosphate of ATP in DltA and ACSM2A shows that its side chain is highly dynamic and flexible. The crucial catalytic role of the invariant A10 lysine residue is strongly supported by the complete activity loss in the adenylation half-reaction without affecting the thioesterification half-reaction in several ANL enzymes when it is mutated (8, 13, 20, 66, 67). It is also supported by the hydrogen bonding interaction of this residue with the alpha-phosphoryl group in complexes of the adenylate-AMP intermediate or analogues with several ANL enzymes, including BsMenE (17, 18, 61, 68) and the use of acetylation of this residue as a regulation control of acetyl-CoA synthetase in mammals (46). Noticeably, the hydrogen bond between this invariant A10 lysine residue and the OSB carboxylate group found in the modeled ATP-Mg<sup>2+</sup>-BS-BsMenE complex should align the substrates for the reaction and put the side chain in the proper position to stabilize the transition state, although it may reduce the nucleophilicity of the attacking carboxylate. With the transition state stabilization by the invariant A10 lysine, the reaction between the two aligned substrates as visualized in the modeled ATP-OSB-BsMenE complex (Fig. 6), should proceed smoothly to form the adenylate intermediate and pyrophosphate.

Noticeably, a structure relaxation mechanism is intrinsically installed in the compact ATP-configured adenylate-forming conformation for its easy disassembly, which is required after adenylation to allow transition to the thioester-forming conformation in the domain alteration mechanism. When the adenylation is complete in the adenylate-forming conformation, the pyrophosphate product is released easily from one end of the ATP-binding tunnel, which is very close to the protein surface (Fig. 2C), to conform to the ordered Bi Uni Bi ping-pong mechanism. This product release eliminates the strong interaction of the A8 Arg-382 to the beta-phosphate of ATP, which controls the correct orientation of the C-domain in the conserved ATP-dependent configuration of the adenylation active site. Consequently, the interdomain interaction is significantly weakened, similar to that observed for the loosened AMP-BsMenE structure (Fig. 2B), to reach a state that allows another conformational change to accept the coenzyme A substrate and form a new active site for the second-step thioesterification. Regaining the C-domain flexibility after adenylation is demonstrated by the two different C-domain orientations found in the OSB-AMP-BsMenE structure (61).

In summary, besides being the first substrate of the ANL enzymes, ATP plays a pivotal structural role in configuring the active adenylating conformational change to impact on the adenylation half-reaction in the ordered substrate binding, the in-line alignment of the two substrates for backside nucleophilic substitution, and stabilization of the transition state. This is achieved by its intense interaction with the P-loop and other structural elements, which are highly conserved in the ANL family. For this conserved mode of ATP binding in configuration of the adenylation active site, it is important to note that the strongest interaction affecting the C-domain orientation occurs at the beta-phosphate of the bound ATP. This mode of C-domain control allows easy disassembly of the adenylate-forming conformation to release the C-domain for a large angle rotation in configuration of the second active site for thioesterification, once the first half-reaction releases its pyrophosphate product to eliminate the domain-affecting interaction. Consequently, the ATP configuration of the adenylate-forming conformation is transient and is intimately coupled to progression of the adenylation half-reaction. Revelation of this structural role of ATP has provided novel mechanistic insights into the adenylation half-reaction and the transition to the thioesterification half-reaction to allow us a better understanding of the domain alteration mechanism of the adenylating enzymes in the ANL family.

Author Contributions—Z. G. conceived and coordinated the study. Z. G. and Y. C. wrote the paper. Y. C. designed, performed, and analyzed experiments in all figures. Y. S. and H. S. participated in protein purification, protein crystallization, data collection, and structural determination. All authors reviewed the results and approved the final version of the manuscript.

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