The mevalonate pathway produces isopentenyl diphosphate (IPP), a building block for polyisoprenoid synthesis, and is a crucial pathway for growth of the human bacterial pathogen Enterococcus faecalis. The final enzyme in this pathway, mevalonate diphosphate decarboxylase (MDD), acts on mevalonate diphosphate (MVAPP) to produce IPP while consuming ATP. This essential enzyme has been suggested as a therapeutic target for the treatment of drug-resistant bacterial infections. Here, we report functional and structural studies on the mevalonate diphosphate decarboxylase from E. faecalis (MDD$_{EF}$). The MDD$_{EF}$ crystal structure in complex with ATP (MDD$_{EF}$–ATP) revealed that the phosphate-binding loop (amino acids 97–105) is not involved in ATP binding and that the phosphate tail of ATP in this structure is in an outward-facing position pointing away from the active site. This suggested that binding of MDD$_{EF}$ to MVAPP is necessary to guide ATP into a catalytically favorable position. Enzymology experiments show that the MDD$_{EF}$ performs a sequential ordered bi-substrate reaction with MVAPP as the first substrate, consistent with the isothermal titration calorimetry (ITC) experiments. On the basis of ITC results, we propose that this initial prerequisite binding of MVAPP enhances ATP binding. In summary, our findings reveal a substrate-induced substrate-binding event that occurs during the MDD$_{EF}$-catalyzed reaction. The disengagement of the phosphate-binding loop concomitant with the alternative ATP-binding configuration may provide the structural basis for antimicrobial design against these pathogenic enterococci.

Multidrug-resistant microorganisms have been found in humans and food animals due to the overuse of antibiotics (1). In the United States, a surveillance of antibacterial resistance from the Centers for Disease Control and Prevention reports the deaths and the cost resulting from infectious diseases and emphasizes the urgency to control drug-resistant bacteria (2). This critical list includes vancomycin-resistant enterococci (VRE), which cause a range of enterococcal infections such as bacteremia, urinary tract infections, intra-abdominal and pelvic infections, central nervous system infections, skin and skin structure infections, and infective endocarditis (2–4).

Enterococci are Gram-positive and facultative anaerobes colonized in the gastrointestinal tract. The first clinical isolates of vancomycin-resistant enterococci were reported in Europe in 1988 (5, 6). The VRE bacteria are intrinsically insensitive to detergents and antibiotics (clindamycin, cephalosporins, and aminoglycosides) and keep gaining drug resistance to other newly invented antibiotics, such as linezolid and daptomycin (7–11). The emergence of drug-resistant enterococci results in difficulties of treatment of VRE infections (12–14). For the protection of patients in health care settings, new approaches and new antimicrobial agents against enterococcal infections are urgently needed.

In 2000, Wilding et al. (15) reported that the mevalonate pathway is pivotal in the Gram-positive bacterial pathogens, enterococci, staphylococci, and streptococci. This pathway includes six enzymes, acetyl-CoA acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate decarboxylase (EC 4.1.1.33), that ultimately produce IPP. IPP then serves as a building block for polyisoprenoid synthesis in living organisms ranging from bacteria to humans (16–18). In bacteria, isoprenoid production is also involved in the biosynthesis of bacterial cell wall and electron carriers in the respiratory chain (16, 19–21).

The five enzymes in the mevalonate pathway (except acetyl-CoA acetyltransferase) have been identified to be critical for bacterial growth (15). Experiments with the mammalian...
In this research, we have studied MDD from Enterococcus faecalis (MDD\textsubscript{EF}) from functional, biophysical, and structural points of view to decipher the substrate-binding mechanism of MDD\textsubscript{EF}. MDD proteins trigger the irreversible ATP-dependent decarboxylation of MVAPP to produce IPP in the last step of the mevalonate pathway (Fig. 1) (24). Several residues (aspartate, lysine, serine and arginine) in the active site of MDD proteins have been identified as being involved in substrate binding and enzyme catalysis (25–27). In 2012, Barta et al. (27) reported several crystal structures of the wild-type and mutant forms of MDD from Staphylococcus epidermidis (MDD\textsubscript{SE}) with or without substrates/analouges binding to the active-site cleft. From their structural models, they proposed that MVAPP is the first substrate to bind to the deeper pocket in the active site of MDD\textsubscript{SE}, where Ser-106, Arg-144, Ser-192, and Arg-193 hold MVAPP in position. Next, ATP sits in the ATP-binding pocket interacting with Ser-94 and Asn-96, and the phosphate tail is clamped by Ser-106, Ser-107, and the phosphate-binding loop (Ala-101, Gly-103, and Leu-104), followed by enzyme catalysis (27). The substrate-binding order of MDD isolated from chicken liver was examined with results that suggest avian MDD performs a sequential ordered bi-substrate mechanism with MVAPP as the first substrate (28, 29). However, no kinetic evidence was presented that prokaryotic MDDs act on substrates in the same manner,. A case in point is the enzyme mechanism of proteins in the GHMP kinase family (galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalinate kinase) superfamily, which catalyzes ATP-dependent reactions. The overall structures of MDD\textsubscript{EF} and MDD\textsubscript{EF}–ATP superimpose well with an r.m.s.d of 0.174 Å. In MDD\textsubscript{EF}, the phosphate-binding loop (97–104) and residues from 183 to 192 are missing. Similarly, in MDD\textsubscript{EF}–ATP, residues from 184 to 189 cannot be determined. However, the phosphate-binding loop can be observed (Fig. 2D). In the literature, the phosphate-binding loop in MDD is known for ATP binding (27). In our crystal structure of MDD\textsubscript{EF}–ATP, the phosphate-binding loop does not interact with the ATP molecule (Fig. 3A), although ATP binds to the ATP-binding pocket via the interactions between ATP and the conserved residues (Ser-93, Asn-95, and Ser-105 (Fig. 3B)).

**Structural differences between MDD\textsubscript{EF}–ATP and MDD\textsubscript{SE}–FMVAPP–ATPγS infer the pivotal role of MVAPP in ATP binding**

By comparing our crystal structure of MDD\textsubscript{EF}–ATP with the tertiary crystal structure of MDD\textsubscript{SE} (MDD\textsubscript{SE}–FMVAPP–ATPγS, PDB code 4DPT) (27), three major differences were found in the phosphate-binding loop (Fig. 3C) and the ATP/ATPγS configurations (Fig. 3D). First, the phosphate-binding loop in MDD\textsubscript{EF}–ATP remains in an open conformation and does not bend down to form contacts with ATP, if compared with the phosphate-binding loop in MDD\textsubscript{SE}–FMVAPP–ATPγS (Fig. 3C). The largest distance moved by the backbone


**Induced substrate-binding mechanism of MDD<sub>EF</sub>**

The crystal structure of MDD<sub>EF</sub>–ATP described above implied that the binding of MVAPP may affect ATP binding. The previous work on avian MDD suggests the substrate-binding order of MDD<sub>EF</sub> from chickens belongs to an ordered bi-substrate mechanism with MVAPP binding first (28). From our crystal structure of MDD<sub>EF</sub>–ATP, ATP can bind to the ATP-binding pocket by itself, even if in an unproductive form (Fig. 3, B and D). Therefore, the question arises whether the substrate-binding order is conserved in both eukaryotic and prokaryotic MDD proteins. To address this issue, we performed kinetics studies intro-
Induced substrate-binding mechanism of MDD$_{EF}$

The patterns in Fig. 4, B and D, show that the kinetic curves in the double-reciprocal plots converge to the x axis. We could then confirm that the enzyme mechanism of MDD$_{EF}$ belongs to a sequential bi-substrate mechanism instead of a ping-pong mechanism or a rapid-equilibrium sequential ordered mechanism. From the curve fitting, we obtain the following kinetic parameters: $V_{\text{max}} = 16.1 \pm 0.3 \mu \text{mol/min/mg}; k_{\text{cat}} = 9.8 \pm 0.2 \text{s}^{-1}; K_m = 39.7 \pm 2.8 \mu \text{M}$; and $K_m = 166 \pm 12 \mu \text{M}$ (Table 2). To determine whether substrates bind to MDD$_{EF}$ in an ordered or random manner, we introduced ATP$\gamma$S into the reaction buffer as a dead-end inhibitor of MDD$_{EF}$ and then determined the substrate-binding order based on the inhibitory patterns of ATP$\gamma$S (supplemental Table S2) (28, 33). Two sets of inhibitory assays were conducted. The first set of experiments was performed at a constant MVAPP concentration of 40 $\mu$M and varying concentrations of MgATP and ATP$\gamma$S (see under “Experimental procedures”). The kinetic data from the inhibitory assays were best represented by a modified competitive inhibition model as shown in Equation 2.

$$
\frac{1}{v} = \frac{1}{0.5 \times V_{\text{max}}} \left(1 + \frac{K_d (1 + K_d / K_A)}{B} + \frac{[I]}{K_d (1 + K_d / K_A)}\right)
$$

(Eq. 2)

where $V_{\text{max}}$ is the maximum velocity; $[B]$ is the varying concentrations of ATP; $K_d$ is $K_{m\text{MgATP}}$; $K_{\text{IA}}$ is $K_{m\text{MVAPP}}$; $K_A$ is $K_{m\text{MVAPP}}$; $K_{\text{IA}} / K_A$ is 0.8, which was derived from the enzymatic reactions described above; $[I]$ is the varying concentration of ATP$\gamma$S, and $K_d$ is $K_{m\text{ATP}\gamma\text{S}}$ (33).

The kinetics data are shown as a Michaelis-Menten plot (Fig. 5A) and a Lineweaver-Burk plot (Fig. 5B). The values of $V_{\text{max}}$, $K_{m\text{MgATP}}$, and $K_{m\text{ATP}\gamma\text{S}}$ were derived from Equation 2 and are summarized in Table 2.

The second set of experiments was performed at a constant MgATP concentration of 200 $\mu$M and varying concentrations of MVAPP and ATP$\gamma$S (“Experimental procedures”). The data set was best represented by a modified uncompetitive inhibition model as shown in Equation 3.

$$
\frac{1}{v} = \frac{1}{0.5 \times V_{\text{max}}} \left(1 + \frac{[I]}{2 \times K_{\text{IA}}} + \frac{K_d (1 + K_d / K_A)}{[A]}\right)
$$

(Eq. 3)
strate-binding mechanism of MDD to be conserved between the eukaryotic and prokaryotic species in the family of MDD proteins.

Isothermal titration calorimetry (ITC) results suggest an MVAPP-induced ATP-binding mechanism of MDDEF. From the kinetics study on MDDEF, MVAPP is identified as the first substrate in the reaction mechanism. Our complex structure of MDDEF–ATP shows that in the absence of MVAPP, ATP binds the ATP-binding pocket in an unusual configuration (Fig. 3, B and D), and the phosphate-binding loop does not bend down to interact with the ATP molecule (Fig. 3C). These results imply the binding of MVAPP may help form a binding site for ATP in its catalytically favored configuration. In other words, the binding of ATP might be enhanced by the prerequisite binding of MVAPP. To test this, we employed thermodynamic approaches and utilized ITC (34) to determine the dissociation constants of the first substrate, MVAPP, the second substrate, MgATP, and the ATP analogues, ATPγS and AMPPCP.

We have determined the $K_d$ values of substrates or ligands (MVAPP, MgATP, AMPPCP, and ATPγS). The raw data for titrations of MDDEF are shown in Fig. 6. All the derived thermodynamic parameters, $K_d$ (1/K_a), are listed in Table 3. The $K_{dMVAPP}$ value is $20.4 \pm 9.3 \mu M$ (Fig. 6A); the $K_{dATP}$ value is $288 \pm 36 \mu M$ (Fig. 6B); and the $K_{dATPγS}$ value is $215 \pm 8 \mu M$ (Fig. 6C). Next, we determined $K_{dATPγS}$ under the condition in which MDDEF was pre-incubated with MVAPP. MDDEF was incubated with 1 mM MVAPP for 30 min to ensure that the MVAPP-binding pocket of MDDEF was nearly fully occupied (about 97.8%), according to the dissociation constant at equilibrium as shown in Equation 4, where [MDDEF]_{total} is the total concentration of MDDEF; [MVAPP]_{total} is the total concentration of MVAPP; and $x$ is the concentration of the MDDEF–MVAPP complex.

$$K_d = \frac{([\text{MDDEF}]_{\text{total}} - x)([\text{MVAPP}]_{\text{total}} - x)}{x} \quad (\text{Eq. 4})$$

Under these conditions, the $K_d$ value between ATPγS and MDDEF–MVAPP was determined to be $25.4 \pm 5.5 \mu M$ (Fig. 6D), which is 10-fold less than the $K_d$ value between ATPγS and MDDEF alone, supporting our proposed model in which the pre-binding of MVAPP would strengthen ATPγS binding. Our
results show that the binding of ATPγS is enhanced by the prerequisite binding of MVAPP to MDD_{EF}, suggesting the binding of MVAPP would trigger conformational changes of MDD_{EF} to accommodate the ATP molecule in its catalytically favored position for the subsequent chemical steps in the reaction.
Discussion

GHMP family kinases employ a variety of mechanisms and enzyme order. Previously, the substrate-binding mechanism of MDD from chickens was determined to belong to a sequential bi-substrate mechanism with MVAPP as the first substrate (28); from our study, we determined prokaryotic MDD from *E. faecalis* also binds MVAPP first during the enzymatic reaction. MDD is characterized as a member GHMK of the kinase family (26). For the galactokinase from pig liver, MgATP is the first substrate of the galactokinase, followed by the binding of galactose (35); however, in *E. coli*, the galactokinase enzyme performs a random bi-substrate mechanism (36). For the homoserine kinase in *E. coli*, MgATP is preferred to bind to the enzyme first (37). For the mevalonate kinase from hog liver, a sequential ordered bi-substrate mechanism is performed with mevalonate binding first (38). The enzyme mechanism of the phosphomevalonate kinase from pigs belongs to a sequential ordered bi-substrate mechanism with phosphomevalonate binding first (39). In the case of the MDD, the enzyme mechanism may be conserved among the family of proteins; however, in other cases in the GHMP kinase family, the enzyme mechanisms may vary in species. Although in this study we aim at
Induced substrate-binding mechanism of MDD_{EF}

elucidating the intriguing observations from the structure of MDD_{EF}-ATP and the structural basis of MVAPP for assisting ATP binding to MDD_{EF}, alternative substrates or product inhibition strategies at both saturated and unsaturated concentrations of MVAPP and MgATP (40) are also the ways to comprehensively investigate the overall enzyme mechanism of MDD_{EF}.

MDD proteins have been known to trigger the ATP-dependent decarboxylation of MVAPP, but with a requirement for metal ions (Mg^{2+}) to perform catalysis under physiological conditions (29). In our crystal structure of MDD_{EF}-ATP, the ATP molecule does not have magnesium binding to the α- and β-phosphate group. In the crystallization condition, we have 5 mM magnesium chloride and 10 mM ATP in the solution. The buffer condition is 50 mM sodium acetate at pH 4.6, and the protonation state of ATP is mainly HATP^{3-} under this condition (41). The dissociation constant between HATP^{3-} and magnesium is about 1.58 mM (log K_{d} = 2.79 ± 0.15) (42), and the MgATP concentration under this condition is about 4 mM, suggesting the concentration of free HATP^{3-} is about 6 mM. The reason for not seeing magnesium-bound ATP in the crystal structure of MDD_{EF}-ATP could be due to the competitive binding of free HATP^{3-} to the ATP-binding pocket of MDD_{EF}. It is also possible that a higher average temperature factor of the structure and function of the MDD family of proteins remains an important but challenging field.

Table 3
Thermodynamic parameters

Titration experiments were done at 25 °C. NBD indicates no detectable binding.

| Species | Substrate | Ka (μM) | ΔG° (kcal/mol) | ΔH (kcal/mol) | ΔS (cal/mol/K) |
|---------|-----------|---------|----------------|---------------|----------------|
| a       | MDD_{EF} (100 μM) + MVAPP (1 mM) | 18.7 ± 7.4 | −6.50 ± 0.23 | 0.91 ± 0.18 | 7.41 ± 0.20 | 24.88 ± 0.69 |
| b       | MDD_{EF} (100 μM) + AMP (1 mM) | 215 ± 8 μM | 21.5 ± 5.8 | 6.70 ± 0.3 | 7.01 ± 0.2 | 24.79 ± 0.69 |
| c       | MDD_{EF} (100 μM) + ATP (3 mM) | 288 ± 36 μM | −4.8 ± 0.1 | −4.8 ± 0.1 | 1.0 ± 0.3 | 3.5 ± 1.0 |
| d       | MDD_{EF} (100 μM) + MVAPP (1 mM) | 215 ± 8 μM | 21.5 ± 5.8 | 6.70 ± 0.3 | 7.01 ± 0.2 | 24.79 ± 0.69 |
| e       | MDD_{EF} (100 μM) + AMP (1 mM) | 271 ± 43 | −4.87 ± 0.09 | −4.87 ± 0.09 | 1.67 ± 0.3 | 5.61 ± 1.21 |
| f       | MDD_{EF} (100 μM) + MVAPP (1 mM) | 288 ± 36 μM | −4.8 ± 0.1 | −4.8 ± 0.1 | 1.0 ± 0.3 | 3.5 ± 1.0 |
| g       | MDD_{EF} (100 μM) + AMP (1 mM) | 215 ± 8 μM | 21.5 ± 5.8 | 6.70 ± 0.3 | 7.01 ± 0.2 | 24.79 ± 0.69 |

Figure 6. Original titration curves from ITC experiments with MDD_{EF}. A, MDD_{EF} (100 μM) titrated with MVAPP (2 mM). B, MDD_{EF} titrated with ATP (3 mM). C, MDD_{EF} titrated with ATPγS (3 mM). D, MDD_{EF} pre-incubated with MVAPP (1 mM) and then titrated with ATPγS (2 mM). The protein concentration is adjusted to 100 μM, and all the protein and titrants are dissolved in the buffer containing 100 mM HEPES, pH 7, 100 mM KCl, and 10 mM MgCl$_2$.
**Induced substrate-binding mechanism of MDD**

The open conformation of the phosphate-binding loop and the unusual configuration of ATP in our crystal structure of MDD$_{EF}$–ATP suggest the importance of MVAPP binding in forming the productive ATP-binding site. We proposed that MVAPP could induce ATP binding to MDD$_{EF}$, and the thermodynamic results are in agreement with our proposal. In addition to the examination of changes of $K_{d}$ values in the presence of excess amounts of MVAPP in the solution, we also tested whether this enhanced binding occurs in the titration experiments using AMP/PCP. The $K_{d}$ value was $271 \pm 43 \mu M$ (Table 3, row e); however, in the presence of MVAPP, we could not detect heat changes and so could not obtain the thermodynamic parameters in this case (Table 3, row f). If instead MDD$_{EF}$ is pre-incubated with excess amounts of AMP/PCP (10-fold, MDD$_{EF}$–AMP/PCP complex formation rate: 77.4%) and then the mixture is titrated with MVAPP (Table 3, row g), the $K_{d}$ value in this case is $18.7 \pm 7.4 \mu M$, which is a similar value as from the MDD$_{EF}$–MVAPP titration (Table 3, row a), indicating AMP/PCP may have little effect on MVAPP binding. We have also done a preliminary test on ATP$_{γ}$S. MDD$_{EF}$ was pre-incubated with excess amounts of ATP$_{γ}$S (10-fold, MDD$_{EF}$–ATP$_{γ}$S complex formation rate: 81.1%) and then the mixture was titrated with MVAPP (data not shown). Although there are no other experimental results to explain the two-process binding, a quick saturation after the 6th injection, suggesting a tight binding event occurs. $K_{d}$MVAPP was roughly determined to be $76 \text{ nm}$ (data not shown). Although there are no other experimental results to explain the two-process binding, a quick saturation after the 6th injection indicates that MVAPP binds MDD$_{EF}$–ATP$_{γ}$S much tighter than MVAPP binds MDD$_{EF}$ alone (Table 3, row a). It also implies that AMP/PCP might not be able to access a catalytically-favored conformation for “locking” MVAPP in the active site, which might need, for example, an aid from the conformational changes of MDD$_{EF}$ upon substrate binding. The conserved phosphate-binding loop of the MDD family of proteins functions to interact with the phosphate tail of ATP. It has been shown that AMP/PCP has poor inhibitory activity ($K_{i} > 1 \text{ mm}$) against MDD from chickens (28) and MDD$_{EF}$ in our preliminary enzymatic study (data not shown). AMP/PCP has a replacement of the bridging oxygen with the methyl group ($\text{CH}_2$) between the $β$- and $γ$-phosphorus atoms. We therefore propose that the $β$-$γ$-bridging oxygen of ATP serves as a key checkpoint for the molecular recognition of ATP by MDD$_{EF}$ during the enzymatic reaction.

To summarize our results, we suggest that an induced substrate-binding mechanism of MDD proteins occurs during the enzymatic reaction (Fig. 7), in which the binding of the first substrate, MVAPP, to MDD will trigger the conformational changes of the enzyme to accommodate ATP binding to the ATP-binding pocket in its catalytically favored position, followed by enzyme catalysis and product release. The observation that ATP can bind by itself but in a catalytically unfavorable position may also provide an alternative configuration of the MDD$_{EF}$ enzyme that provides a new structural basis for antibacterial design for pathogenic enterococci.

![Figure 7. Induced substrate-binding mechanism of MDD proteins. Left panel (green), apo-form of MDD in which two pockets for substrate binding are empty; middle panel (orange), the MVAPP-bound MDD in which the binding of MVAPP (shown in a red triangle) triggers conformational changes of the enzyme and reshapes the ATP-binding pocket, which allows the binding of ATP (shown in a blue arrow) to its catalytically favored position; right panel (purple), two-substrate-bound MDD. Step 1, the binding of MVAPP; step 2, the binding of ATP; step 3, enzyme catalysis and product release. Products in different shapes are shown in gray.](image)

**Cloning, overexpression, and purification of the recombinant form of MDD$_{EF}$**

A gene fragment encoding MDD from *E. faecalis* (MDD$_{EF}$) was amplified via PCR and subcloned into the expression plasmid pET30a (43). Upon confirmation of the DNA sequence, the construct was transformed into *E. coli* BL21 (DE3). Transformed cells were cultured in LB broth supplemented with kanamycin (50 mg/ml) at 37 °C to an $A_{600}$ nm of 1.0. Protein expression of MDD$_{EF}$ was induced by addition of isoprrophy 1-thio-$β$-$D$-galactopyranoside (0.1 mm) for another 4 h at 37 °C.

Cells were harvested by centrifugation at 8000 rpm, resuspended in binding buffer (50 mm sodium phosphate, pH 7.4, 300 mm NaCl, and 10 mm imidazole), and lysed to homogeneity by a French press. His-tagged MDD$_{EF}$ protein was soluble in the supernatant after centrifugation and trapped on a Ni$^{2+}$-NTA column followed by the elution with increasing concentrations of elution buffer (50 mm sodium phosphate, pH 7.4, 300 mm NaCl, and 300 mm imidazole). Eluted fractions were pooled and desalted against dialysis buffer (25 mm Tris-HCl, pH 8.0, 100 mm NaCl, and 10 mm MgSO$_4$) twice, the first time with $β$-mercaptoethanol (2-ME) (20 mM) and the second time without 2-ME. The N-terminal His tag was removed from MDD$_{EF}$ by treatment with recombinant TEV protease in dialysis buffer containing 1 mm DTT and 0.5 mm EDTA overnight at room temperature for 4 h followed by a final dialysis procedure at 4 °C in the dialysis buffer without DTT and EDTA. His-tagged TEV and residual His-tagged MDD$_{EF}$ were removed by passing the protein mixture through a nickel affinity resin. The artificial sequence NA remains at the N terminus of MDD$_{EF}$ after the TEV treatment. The purified MDD$_{EF}$ protein solution was concentrated to 8–10 mg/ml by ultrafiltration and stored at $−20$ °C.

**Protein crystallization**

The co-crystal of MDD$_{EF}$ in complex with ATP (MDD$_{EF}$–ATP) grew under the crystallization condition (10 mm ATP, 26% PEG 3350, 50 mm sodium acetate, pH 4.6, and 5 mm MgCl$_2$) by sitting drop vapor diffusion. ATP was added to the
protein solution to a final concentration of 10 mM. 1 μl of MDD\textsubscript{EF}-ATP solution was mixed with 1 μl of the reservoir. Crystals formed after a 2-day equilibrium period. Cryo-protectant (26% PEG 3350, 17% PEG 400, 50 mM sodium acetate, pH 4.6, and 5 mM MgCl\textsubscript{2}) was prepared and gradually added into the protein/reservoir mixture to prevent ice formation when flash-freezing the crystal in liquid nitrogen.

A stable condition (1.6 mM ammonium sulfate, 50 mM sodium acetate, pH 4.6) for growing crystals of the apo-form of MDD\textsubscript{EF} was obtained and optimized from a commercialized crystallization screening kit (Qiagen, class II, condition A2: 0.1 mM sodium acetate, pH 4.5, 2 mM ammonium sulfate). Protein solution (8 to 9 mg/ml) was mixed with the reservoir solution at a 1:1 ratio and equilibrated by vapor diffusion of sitting drops at 20 °C for 2 days. In a 96-well plate, there were about 20 wells containing single cuboid crystals with a size of 0.2 to 0.3 mm. Cryo-protectant (25% glycerol, 1.6 mM ammonium sulfate, 50 mM sodium acetate, pH 4.6) was prepared and gradually added into the protein/reservoir mixture to prevent ice formation when flash-freezing the crystal in liquid nitrogen.

**Data collection and structure determination, refinement analysis, and refine statistics**

The diffraction data from crystals of MDD\textsubscript{EF} co-crystallized with ATP (MDD\textsubscript{EF}-ATP) and an apo-form of MDD\textsubscript{EF} (MDD\textsubscript{EF}) were collected at the 23-ID-D and 19-ID-BM beamlines at the Advanced Photon Source (APS) at Argonne National Laboratory in Chicago. The HKL2000 software was used for data integration, data reduction, and data scaling (44), generating a scale pack reflection file. The space group of the used for data integration, data reduction, and data scaling (44), generating a scale pack reflection file. The space group of the crystal was determined as P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} (MDD\textsubscript{EF}-ATP: a, b, c = 82.0, 97.7, 46.3 Å; α, β, γ = 90°; MDD\textsubscript{EF}: a, b, c = 81.9, 98.1, 46.1 Å; α, β, γ = 90°) by the software Pointless in CCP4 and phenix.phaser (45, 46). The software in CCP4, scalepack2mtz, was then used to convert the scale pack reflection file (.sca) to an MTZ format (.mtz) with R-free flag assigned (5–5.5%) (47).

One molecule was expected in an asymmetric unit by analyzing the Matthews coefficient and the water content in each case (MDD\textsubscript{EF}-ATP: cell volume: 370710.5 Å\textsuperscript{3}, molecular mass: 36489.5 Da, Matthews coefficient: 2.54, % solvent: 51.6; MDD\textsubscript{EF}: cell volume: 370,279.3 Å\textsuperscript{3}, molecular mass: 36489.5 Da, Matthews coefficient: 2.54, % solvent: 51.6). Phenix.phaser was used to solve the phases and determine the structure of MDD\textsubscript{EF}, MDD\textsubscript{EF} and MDD\textsubscript{EF} share 60% sequence identity. A modified search model (polyalanine) generated from MDD from *S. epidermidis* (MDD\textsubscript{SE}, PDB code 3QT5) using CHAINSAW in CCP4 (48) was used to produce initial phases for the co-crystal structure of MDD\textsubscript{EF}-ATP in molecular replacement with phenix.phaser (45). In each step of molecular replacement, only one solution was found. The rotation function with likelihood gain greater than 0, the translation function with Z-score greater than 8, and no violations after the packing analysis also suggest the success of the molecular replacement approach. The phases for determining the crystal structure of the apo-form of MDD\textsubscript{EF} were produced by molecular replacement with Phenix.phaser (45) using a polyalanine-modified MDD\textsubscript{EF}-ATP structure (described above) as a search model.

After phase determination, the structure refinement was performed in phenix.refine (45). During structure refinement, strategies for refining the model geometry (XYZ coordinates), atomic positions (Real space), and atomic B-factors (individual B-factors) were chosen, and after the refinement, the missing side chains of the residues in the structural model were manually rebuilt in the graphics program Coot (49) based on observation of the electron density map (2Fo – Fc) and the difference map (Fo – Fc). The simulated-annealing (Cartesian) option was also employed in the first few runs of structure refinement. In phenix, we used the phenix.composit_omit_map software to generate the omit map and used the maps to evaluate the refined structural models. Target function optimization was also chosen for refining the weight between X-ray data and the structural model (optimize X-ray/stereochemistry weight, optimize X-ray/ADP weight). Water molecules were built by either phenix.refine or Coot and inspected in the Coot interface.

The crystallographic information file (.cif) and the PDB format file (.pdb) of ATP were generated using phenix.eLBOW (45). After a few runs of structure refinement without ligands, ATP was manually placed and fitted into the weighted difference electron density maps in Coot (49). Geometry and rotamer outliers were inspected and evaluated after each structure refinement. Finally, ATP was omitted by setting the ligand occupancy to zero or simply removing it from the structure for calculating a simulated-annealing-ligand-omit map in phenix.composite_omit_map, and the ligand-omit map and its corresponding structure were examined in PyMOL (50).

**Enzyme kinetics of wild-type MDD\textsubscript{EF}**

Enzymatic activity of MDD\textsubscript{EF} was determined using an ATP/NADH enzyme-coupled assay. In this assay, ADP produced from the MDD reaction is converted back to ATP by pyruvate kinase, coupled with the conversion of phosphoenolpyruvate to pyruvate, and the pyruvate is then converted to lactate by lactate dehydrogenase, coupled with the oxidation of NADH to NAD\textsuperscript{+}. The oxidation of NADH to NAD\textsuperscript{+} results in an absorbance decrease at 340 nm. Thus, the ADP production can be detected by measuring the decrease in A\textsubscript{340 nm}. The MDD enzymatic activity would be proportional to the slope of the continuously declining value of A\textsubscript{340 nm}. We have utilized this method to determine the enzymatic activity of MDD\textsubscript{EF} under different conditions for obtaining kinetic parameters of MDD\textsubscript{EF}. Each reaction was performed at 30 °C under the buffer condition (100 mM HEPES, pH 7.0, 100 mM KCl, 10 mM MgCl\textsubscript{2}, 0.2 mM NADH, 0.4 mM phosphoenolpyruvate, 4 units of pyruvate kinase, 4 units of lactate dehydrogenase, and 100 mM MDD\textsubscript{EF}) (27).

Initial velocity of each reaction was determined at a range of concentrations of MgATP (50, 100, 150, 200, 400, 600, 800, and 1000 μM) and MVAPP (10, 15, 25, 50, 100, 150, 200, and 300 μM). Final enzymatic parameters, K\textsubscript{m} and V\textsubscript{max} were determined by fitting kinetic data to a sequential bi-substrate mechanism model using SigmaPlot12.5/Enzyme Kinetic Module 1.3 (Systat Software, Inc.).
Induced substrate-binding mechanism of MDD_{EF}

Inhibition assays

ATPγS was used as a competitive inhibitor of ATP for determining the inhibition kinetics of MDD_{EF}. Different fixed concentrations of ATPγS (0, 100, 200 and 400 μM) were added into the reactions versus varying MgATP (50, 100, 200, and 400 μM) and fixed MVAPP (40 μM) or varying MVAPP (10, 20, 40, 80 μM) and fixed MgATP (200 μM). Assays were performed under the conditions as described above. The kinetic data with a fixed MVAPP concentration were fit with a competitive inhibition model; the kinetic data with a fixed MgATP concentration were fit with an uncompetitive inhibition model using SigmaPlot as described previously.

ITC experiments

The preparation of TEV-treated MDD_{EF} was described above. The protein solution was dialyzed against the same buffer as used in the enzymatic reactions described previously (100 mM HEPES, pH 7.0, 100 mM KCl and 10 mM MgCl₂). All the buffer solutions in ITC experiments were filtered through an 0.45-μm filter and degassed for 1 h at room temperature. The protein concentration was adjusted to 100 μM (260 μl). Each ligand (MVAPP, ATP, AMPPCP, and ATPγS) was prepared in the same dialysis buffer to avoid buffer mismatch. The ITC instrument, MicroCal iTC200, was employed for isothermal titrations in this study, and the reference cell was filled with the instrument source X-ray data collection and the Advanced Photon Source (APS) at Argonne National Laboratory in Chicago for access to the beam lines 19-BM-D and 23-ID-D that contributed to the results presented here.

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Inhibition assays

ATPγS was used as a competitive inhibitor of ATP for determining the inhibition kinetics of MDD_{EF}. Different fixed concentrations of ATPγS (0, 100, 200 and 400 μM) were added into the reactions versus varying MgATP (50, 100, 200, and 400 μM) and fixed MVAPP (40 μM) or varying MVAPP (10, 20, 40, 80 μM) and fixed MgATP (200 μM). Assays were performed under the conditions as described above. The kinetic data with a fixed MVAPP concentration were fit with a competitive inhibition model; the kinetic data with a fixed MgATP concentration were fit with an uncompetitive inhibition model using SigmaPlot as described previously.

ITC experiments

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Author contributions—C. C.-L. and C. V. S. designed the experiments. J. C. M. cloned the MDD protein. C. C.-L. purified the MDDEF protein for the study on enzymology, thermodynamics, and crystallization. C. C.-L. analyzed data and determined the substrate-binding order and the crystal structures of the target protein. L. N. P. designed and performed the ITC experiments. C. N. S. assisted in solving the MDD X-ray crystal structure. C. C.-L. and C. V. S. collaborated in writing the article.

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**Induced substrate-binding mechanism of MDD_EF**