Structure and Mechanism of 2-C-Methyl-D-erythritol 2,4-Cyclodiphosphate Synthase

AN ENZYME IN THE MEVALONATE-INDEPENDENT ISOPRENOID BIOSYNTHETIC PATHWAY*

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Stéphane B. Richard‡, Jean-Luc Ferrer§, Marianne E. Bowman‡, Antonietta M. Lillo¶, Charles N. Tetzlaff¶, David E. Cane†, and Joseph P. Noel‡,‡,§

From the ‡Structural Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037, the §Institut de Biologie Structurale, 38027 Grenoble Cedex 1, France, and the ¶Department of Chemistry, Brown University, Providence, Rhode Island 02912

The enzyme 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MECDP) synthase catalyzes the conversion of 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate (CDP-ME2P) to MECDP, a highly unusual cyclodiphosphate-containing intermediate on the mevalonate-independent pathway to isopentenyl diphosphate and dimethylallyl diphosphate. We now report two X-ray crystal structures of MECDP synthase refined to 2.8 Å resolution. The first structure contains a bound Mn2⁺ cation, and the second structure contains CMP, MECDP, and Mn²⁺. The protein adopts a homotrimeric quaternary structure built around a central hydrophobic cavity and three externally facing active sites. Each of these active sites is located between two adjacent monomers. A tetrahedrally arranged transition metal binding site, potentially occupied by Mn²⁺, sits at the base of the active site cleft. A phosphate oxygen of MECDP and the side chains of Asp⁸, His⁴⁰, and His⁴² occupy the metal ion coordination sphere. These structures reveal for the first time the structural determinants underlying substrate, product, and Mn²⁺ recognition and the likely catalytic mechanism accompanying the biosynthesis of the cyclodiphosphate-containing isoprenoid precursor, MECDP.

The five essential steps of the deoxyxylulose/2-C-methyl-D-erythritol 4-phosphate (DXP/MEP) pathway for isoprenoid biosynthesis have been elucidated and reviewed (1). The terminal steps of this pathway possibly containing a branching point (2, 3) have been attributed to the participation of two additional enzymes, GcpE and LytB (4–10). This pathway, found in many bacteria, the plastids of plant cells, and the apicomplexan parasites such as the malarial causative agents Plasmodium spp. and the opportunistic human pathogen Toxoplasma gondii, leads to the formation of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (11, 12) (Fig. 1). In some bacteria including Archaea, as well as fungi, and the cytoplasm and mitochondria of plants and animals, IPP and DMAPP are produced by the classical acetate/mevalonate pathway (11, 13). IPP and DMAPP represent the C₅ building blocks utilized by all organisms for the biosynthesis of larger isoprenoids of both primary and secondary metabolism (14, 15). Since vertebrates synthesize isoprenoid precursors using a mevalonate-dependent pathway, enzymes of the mevalonate-independent pathway for isoprenoid production represent attractive targets for the structure-based design of selective antibacterial, herbicidal, and antimalarial drugs.

The fifth step of the DXP/MEP pathway involves the highly unusual enzymatic formation of a cyclodiphosphate-containing metabolite, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MECDP). MECDP synthase (16, 17) encoded by the isfg open reading frame of Escherichia coli (formerly named ygbB) catalyzes the formation of MECDP from CDP-ME2P in a Mg²⁺/Mn²⁺-dependent reaction. In this report, we describe the three-dimensional structures of E. coli MECDP synthase bound to Mn²⁺ and the reaction products CMP and MECDP. This study precisely defines the active site features utilized for substrate recognition and catalytic formation of MECDP.

EXPERIMENTAL PROCEDURES
Expression and Purification—The E. coli isfg gene (formerly ygbB, GenBank™ accession number AF230738) encoding MECDP synthase was PCR-amplified from E. coli K12 and inserted into the pHISS expression vector (18). N-terminal His₈-tagged protein was expressed in BL21(DE3) cells. Tagged MECDP synthase was purified from sonicates using a Ni²⁺-nitrilotriacetic acid (Qiagen) column. The histidine tag was removed using thrombin, and the cleaved synthase was purified to greater than 99% homogeneity by gel filtration chromatography on a Superdex-200 (Amersham Biosciences, Inc.) FPLC column. MECDP synthase-containing fractions were combined and concentrated to 1.5 mg ml⁻¹. Selenomethionine (SeMet)-substituted protein was obtained from E. coli grown in minimal medium using the methionine pathway inhibition approach described by Doublé (19) and purified as described above for the native protein.

ESRF, European Synchrotron Radiation Facility; MAD, multiple-wavelength anomalous diffraction; SeMet, selenomethionine; GPP, geranyl diphosphate; FIP, French Beamline for Investigation of Proteins.
isoprenoid biosynthesis. The first step of the DXP/MEP pathway is then phosphorylated in an ATP-dependent reaction by the enzyme DXP reductoisomerase (39), which is subsequently cytidy-catalyzed by the enzyme DXP synthase (36, 37) and appears to be a key step in the formation of DXP from pyruvate and glyceraldehyde-3-phosphate.

The mevalonate-independent or DXP/MEP pathway for the biosynthesis of farnesyl pyrophosphate (FPP) was elucidated in 1989 (38). This pathway is mediated by the enzyme DXP synthase (16) catalyzes the formation of MECDP. Additional steps later in the reaction sequence (4–10).

**Mechanism of the Enzyme MECDP Sythase**

**Data Collection and Processing**—Multiple-wavelength anomalous diffraction (MAD) data were collected at the selenium edge, measuring 3.5–4.0 Å resolution. 5% of the reflections were randomly selected to provide experimental phases. MAD data were processed with 'adp', a script for Automated Data Processing (20). All data were indexed, integrated, and scaled using DENZO/SCALEPACK (21). Intensities were transformed into amplitudes using TRUNCATE (22) (Table I).

**Structure Determination, Model Building, and Refinement**—The crystal structure of MECDP synthase was solved using a MAD data set collected on FIP with the SeMet-substituted protein. Selenium positions were determined using Shake-and-Bake (23). A fourth site was added later after inspection of the sharpened anomalous difference Patterson map. Experimental phases were calculated to 3.5-Å resolution and then improved and extended by solvent flattening to 3.0-Å resolution. MAD phasing and density modification with CNS (24) generated a readily interpretable electron density map. All subsequent refinement steps were carried out with CNS (24). Model building was carried out with the program O (25) using SIGMAA-weighted 2Fo - Fc and Fc - Fo electron density maps (26) and 3Fo - 2Fc electron density omit maps. Refinement consisted of iterative cycles of simulated annealing, positional refinement, and B-value refinement. The initial rounds of building and refinement included residues 1–60 and 72–153 of MECDP synthase and were accomplished using the original MAD data set extending to 3.0-Å resolution.

**Synthesis of MECDP**—A mixture of MEP (522 μmol), CTP (590 μmol), ATP (590 μmol), MgCl2 (73 μmol), Tris-HCl, pH 7.8 (730 μmol), NaOH (1.9 μmol), N,N-dithiothreitol (7.3 μmol), DXP-ME synthetase (E. coli, 40 units), inorganic pyrophosphatase (67 units), CDP-ME kinase (E. coli, 6 mg), and MECDP synthase (E. coli, 6 mg) was incubated for 20 h at 37 °C in a total volume of 7.3 ml. Approximately 80% of the MEP was converted to MECDP as determined by 1H NMR analysis using acetone as an internal standard. The crude reaction mixture was deproteinized (10,000 nominal molecular weight limit filter, Millipore), lyophilized, and then dissolved in 67% (v/v) cold ethanol. The unreacted CTP and ATP together with the products CMP and ADP formed a white precipitate, which was removed by centrifugation (6000 rpm, 15 min). The supernatant was brought up to 22 ml with water and loaded onto an ion exchange column (hand packed Chromabond SB-LV, 2.5 × 150 mm, Macherey & Nagel). The column was eluted with a linear gradient of aqueous ammonium formate (0.1-1.0 M) over a total volume of 240 ml at a flow rate of 3 ml min⁻¹. The eluate was monitored with a linear gradient of aqueous ammonium formate (0.1-1.0 M) over a total volume of 240 ml at a flow rate of 3 ml min⁻¹. The eluate was monitored with a linear gradient of aqueous ammonium formate (0.1-1.0 M) over a total volume of 240 ml at a flow rate of 3 ml min⁻¹.

**Crystallization**—Crystals of MECDP synthase were obtained by the vapor diffusion method at 4 °C in 2-μl hanging drops containing a 1:1 mixture of a 13.5 mg ml⁻¹ protein solution with crystallization buffer (0.8 M (NH₄)₂SO₄, 1% (w/v) polyethylene glycol 400, 0.2 M sodium iodide, 2 mM N,N-dithiothreitol, 0.1 M PIPES, pH 6.5). Crystals appeared after several days as regular hexagons of approximate dimensions 300 × 100 μm. These crystals were stabilized in the presence of the same crystallization buffer containing up to 17.5% (v/v) ethylene glycol as a 30:15:5 mixture and stained with anisaldehyde:ethanol: sulfuric acid using a 4:2:94 mixture. MECDP (retention time 45 min, 1H and 31P NMR analysis) was obtained in 46% yield based on 1H NMR analysis using acetone and methanol as internal standards with no detectable impurities. Negative ion electrospray ionization-mass spectrometry: calculated for [M - H] C₁₂H₁₇O₄P₂, 276.9878; observed, 276.9922.

**Structure Determination, Model Building, and Refinement**—The crystal structure of MECDP synthase was solved using a MAD data set collected on FIP with the SeMet-substituted protein. Selenium positions were determined using Shake-and-Bake (23). A fourth site was added later after inspection of the sharpened anomalous difference Patterson map. Experimental phases were calculated to 3.5-Å resolution and then improved and extended by solvent flattening to 3.0-Å resolution. MAD phasing and density modification with CNS (24) generated a readily interpretable electron density map. All subsequent refinement steps were carried out with CNS (24). Model building was carried out with the program O (25) using SIGMAA-weighted 2Fo - Fc and Fc - Fo electron density maps (26) and 3Fo - 2Fc electron density omit maps. Refinement consisted of iterative cycles of simulated annealing, positional refinement, and B-value refinement. The initial rounds of building and refinement included residues 1–60 and 72–153 of MECDP synthase and were accomplished using the original MAD data set extending to 3.0-Å resolution. 6% of the reflections were randomly selected to provide
**Mechanism of the Enzyme MECDP Synthase**

### RESULTS AND DISCUSSION

**Monomer Structure**—The MECDP synthase monomer spans 159 residues, and the polypeptide chain folds into a single domain. Each polypeptide of the homotrimer organizes around a central, four-stranded β-sheet that packs against three α-helices, two 3_10 helices, and an extended two-stranded antiparallel β-sheet (Fig. 2A). This β-sheet, located between β1 and α1, contains a small two-stranded antiparallel β-sheet (β2 and β3) that folds back against the monomeric subunit near the upper N-terminal side of the α-helix bundle. This extra motif, or “flap,” is structurally similar to the extended “arm” involved in a key intersubunit domain swap in the structure of CDP-ME synthase (30). While the flap does not participate in the formation of the homotrimer, it may be involved in the formation of a larger multienzyme complex by mediating protein-protein interactions among the enzymes of the DXP/MEP pathway.

β1, β4, and β5 of the main β-sheet, composed of residues 2–11, 90–100, and 127–132, respectively, run parallel, while β6, located between β1 and β4, runs antiparallel. Helices α1 and α2 are connected by a 21-residue loop that is not well defined in the calculated electron density maps. Clear density accounting for CMP, MECDP, and a tetrahedrally coordinated metal ion extend across two adjacent monomers and bracket β1 (Fig. 2B).

**Quaternary Architecture**—A number of hydrodynamic analyses including gel filtration chromatography, analytical ultracentrifugation, and static light scattering demonstrate equilibrium of monomeric, trimeric, and hexameric states of MECDP synthase with the trimeric form predominating (data not shown). Analysis of the crystal packing clearly revealed the homotrimeric form of the enzyme (Fig. 2A). The quaternary fold exhibits a modular construction of three units that pack into a circular assembly with perfect 3-fold symmetry (Fig. 2C). The β-sheet motif of one monomer is packed against the related β-strands of the two adjacent monomers, forming a three-bladed propeller-like arrangement (Fig. 2C). This structure creates a central triangular barrel with the β-strands of the barrel approximately parallel to the crystallographic 3-fold axis. The nine remaining α-helices of the trimer enclose the symmetric β-barrel. Similar quaternary arrangements observed previously include the E. coli YjgF protein (31), the YabJ protein (32), chorismate mutase (33), and holoacyl carrier protein synthase from Bacillus subtilis (34). In nearly all cases

### Table I

**Crystallographic data, phasing, and refinement statistics**

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<tr>
<th></th>
<th>A1 (max F^2)</th>
<th>A2 (min F^2)</th>
<th>A3 (remote)</th>
<th>MECDP</th>
<th>Apo</th>
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<td>4.5 (4.5)</td>
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<tr>
<td>Completeness^{a,b} (%)</td>
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<td>99.8 (100)</td>
<td>99.8 (100)</td>
<td>99.3 (100)</td>
<td>93.6 (96.9)</td>
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**Phasing power**

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<td>R_{core}^{d}</td>
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| Phasing power | 0.5170  | 0.5609   | 0.6214

**Quality of the Model**

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<td>Water</td>
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### Footnotes

^{a} For the SeMet data sets, F^+ and F^- were considered nonequivalent when calculating the number of unique reflections and completeness.

^{b} Number in parentheses is for the highest resolution shell.

^{c} R = \sum |F_{o}-F_{c}|/\sum |F_{c}|, where F_{o} and F_{c} are observed and calculated structure factors, respectively.

^{d} R_{core} = \sum |E| \sum |F_{o}|/\sum |F_{c}|, where summation is over the data used for refinement.

^{e} Phasing power = \langle F_{PH}-F_{calc} \rangle/\langle F_{calc} \rangle, where FPH = calculated heavy-atom structure factor and Fcalc is the estimated phase.

^{f} FOM is figure of merit, where FOM = cosine of likely error in phase angles.

^{g} R_{restr} = \sum |E| \sum |F_{o}|/\sum |F_{calc}|, where summation over the data used for refinement.

^{h} R_m = \sum |F_{o}-F_{calc}|/\sum |F_{calc}|, where summation is over the data used for refinement.

^{i} R_{m} = \sum |E| \sum |F_{o}|/\sum |F_{calc}|, where summation over the data used for refinement.

^{j} R_{m} = \sum |E| \sum |F_{o}|/\sum |F_{calc}|, where summation is over the data used for refinement.

^{k} Missing residues | 3 | 4 |

^{l} Protein | 58.9 | 60.3 |

^{m} Water | 54.9 | 56.1 |

^{n} Ligand | 89.5 | None |
including the present study of MECDP synthase, this quaternary arrangement is functionally essential since each of the three active sites are formed by separate polypeptide chains participating in formation of the homotrimeric structure.

A network of complementary hydrophobic contacts, electrostatic interactions, and specific hydrogen bonds stabilize the circular structure between close-packed monomers. His\(^{10}\) and Arg\(^{2}\), located on β1 extend outward from one monomer and link via a salt bridge to Glu\(^{135}\) positioned on the end of β5 and Asp\(^{126}\) situated on a 3\(_{10}\) helix of the adjacent monomer. The side chains of Arg\(^{142}\), Glu\(^{144}\), Phe\(^{139}\), Thr\(^{140}\), Ile\(^{99}\), Phe\(^{7}\), Glu\(^{149}\), His\(^{5}\), Val\(^{151}\), Ile\(^{154}\), and Met\(^{1}\) organize along the interface separating the individual monomers (Fig. 2D). Notably Glu\(^{149}\) and His\(^{5}\), from β6 and β1, respectively, associate at the bottom of the trimeric β-barrel through a complex salt bridge linking the three monomers together.

The Intermonomeric Cavity—The top two-thirds of the trimeric barrel form a spacious cavity into which protrudes a set of largely hydrophobic side chains including Phe\(^{139}\), Thr\(^{140}\), Ile\(^{99}\), and Phe\(^{7}\) (Fig. 2D). The cavity maintains an ellipsoidal shape with the widest part at the equatorial position (16 Å wide). The cap of the intersubunit cavity opens at the top but narrows due to a crown of symmetric salt bridges involving Arg\(^{142}\) and Glu\(^{144}\) resulting in a 5-Å-diameter aperture (Fig. 2, C and D). The bottom of the cavity is closed off by a second complex salt bridge composed of the three glutamate (Glu\(^{149}\)) and three histidine (His\(^{5}\)) residues mentioned in the previous section (Fig. 2D). All of these residues are highly conserved in the MECDP synthase family, and they provide functional support for the trimeric form observed in the crystal.

Notably additional electron density occupies the intermonomeric cavity (Fig. 2D). The entrance to the hydrophobic barrel appears to be capped by a sulfate or phosphate moiety that is sequestered by symmetric interactions with the δ-guanido side chains of Arg\(^{142}\) from each monomer. This electron density extends inward through the hydrophobic core of the cavity along the crystallographic 3-fold axis. Positive identification of the source of this additional electron density is difficult to ascertain from the structure due to the presence of the crystallographic axis and the moderate resolution of the structure. While chemical identification of the origin of this density is currently underway using redissolved crystals, a scenario con-

with CMP, MECDP, and Mn\(^{2+}\). Monomer A pictured on the right is shown with β-strands in green and α-helices in yellow, while monomer B pictured on the left is colored maroon. Monomer C is located in the back, depth cued by fogging, and colored light gray. The CMP and MECDP molecules and the Mn\(^{2+}\) ion are rendered as stick and ball models, respectively. The disordered L1 loop is shown as a dotted line. B, stereo view of the SIGMAA (26) weighted [2\(F_o\) − \(F_c\)] electron density map contoured at 1.0 σ around the CMP:MECDP:Mn\(^{2+}\) complex and calculated at 2.8-Å resolution. C, view of MECDP synthase down a crystallographic 3-fold axis coincident with the 3-fold axis of the homotrimer and marked by a black triangle. The overall orientation of MECDP synthase depicted here is derived from the view shown in panel A following a 90° rotation around the horizontal axis. Arg\(^{142}\) and Glu\(^{144}\) are linked through a ring of salt bridges. D, cross-section view of the central intermonomeric cavity shown in the same orientation as panel A with the 3-fold crystallographic axis vertically oriented. The SIGMAA (26) weighted [2\(F_o\) − \(F_c\)] electron density map contoured at 1.0 σ is represented as a transparent light-blue object. The main chain of the trimer is shown in dark blue, and the side chains of polar residues Arg\(^{142}\), Glu\(^{144}\), Glu\(^{149}\), and His\(^{5}\) and hydrophobic residues Phe\(^{139}\), Thr\(^{140}\), Ala\(^{147}\), Ile\(^{99}\), and Phe\(^{7}\) are rendered and labeled. A GPP molecule, represented in ball-and-stick mode, has been modeled in the extra electron density located at the center of the cavity. The diphosphate part of the GPP molecule interacts with the side chains of three Arg\(^{142}\) δ-guanido side chains, while its hydrocarbon tail resides in the hydrophobic cavity. The bottom of the cavity is pinched off by a complex salt bridge involving Glu\(^{149}\) and His\(^{5}\). (A), (B), and (C) designate each monomer.
sistent with the chemical environment of the intermonomeric cavity can be offered. The shape of the experimentally determined electron density and the local chemical environment of the cavity are consistent with the sequestration of a molecule possessing a negatively charged head group and a hydrophobic tail such as an isoprenoid diphosphate-like molecule (Fig. 2D). Isopentenyl diphosphate (C\textsubscript{5}-IPP), dimethylallyl diphosphate (C\textsubscript{5}-DMAPP), geranyl diphosphate (C\textsubscript{10}-GPP), and farnesyl diphosphate (C\textsubscript{15}-FPP) can all be nicely accommodated in the observed electron density lining the cavity with the optimal fit provided by GPP (Fig. 2D). The possible regulatory and catalytic roles for such a binding event are currently under examination.

**Active Site Architecture**—A key element of the current study was the successful structural elucidation of MECDP synthase bound to the reaction products CMP and MECDP, thus affording the location of active site residues. The three active sites reside at the interfaces of adjacent monomers providing mechanistic relevance to the formation of trimeric MECDP synthase. Residues contributing to the nucleoside-binding portion of the active site originate from the loop linking /H\textsubscript{9252} and /H\textsubscript{9251}. The pyrimidine base of the CMP molecule contacts the enzyme through main chain interactions with Ala\textsuperscript{100}, Pro\textsuperscript{103}, Met\textsuperscript{105}, and Leu\textsuperscript{106} of monomer A. The ribose sugar reaches across to monomer B and forms hydrogen bonds between the carboxyl group of Asp\textsuperscript{56} of monomer B and the 2'- and 3'-hydroxyl moieties of the sugar. Finally the a-phosphate of CMP reaches back to monomer A and forms two hydrogen bonds with the backbone and side chain of Thr\textsuperscript{133} of monomer A (Fig. 3A).

The side chains of Asp\textsuperscript{8}, His\textsuperscript{10}, and His\textsuperscript{42} of monomer B and a nonbridging phosphate oxygen of MECDP tetragonally coordinate a putative cation at the base of the active site (Fig. 3, A and B). Divalent cations such as Mg\textsuperscript{2+} and Mn\textsuperscript{2+} are required for catalytic activity of MECDP synthase (16, 17). In these studies, the highest activities were measured using Mg\textsuperscript{2+} or Mn\textsuperscript{2+}, but other transition metal cations supported catalysis (35). While Mg\textsuperscript{2+} was included prior to and during crystallization, the geometry of the coordination sphere and the soft nature of the coordinating side chains suggest transition metal binding. Initial examination of the metal content of the purified protein by induced coupled plasma atomic emission spectroscopy supports the presence of Mn\textsuperscript{2+} and Ni\textsuperscript{2+}, both of which can form tetragonal coordination complexes. The presence of Ni\textsuperscript{2+} most likely arises during purification, while Mn\textsuperscript{2+} is probably carried along from the *E. coli* expression system. The MECDP binding site extends across monomer B and utilizes both stereospecific hydrogen bonds and van der Waals interactions for sequestration by monomer B and its associated transition metal binding site (Fig. 3B).

**Catalytic Mechanism**—The positions of the reaction products CMP and MECDP in the enzyme active site allow us to model the position of the substrate CDP-ME2P in the intermonomeric catalytic site (Fig. 3C). Furthermore, this model suggests a

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**Fig. 3.** Schematic and structural view of the MECDP synthase active site. A, schematic representation of the CMP, MECDP, and Mn\textsuperscript{2+} binding sites in MECDP synthase. Hydrogen bonds and coordination bonds are shown as dashed green lines. B, rendered view of the MECDP synthase active site. C, putative CDP-ME2P complex and the proposed catalytic pathway for MECDP formation. The CDP-ME2P molecule is fixed by four points binding including a phosphate oxygen-metal ion coordination bond, hydrogen bonds between the C-3 and C-1 hydroxyl groups and the side chains of His\textsuperscript{42} and His\textsuperscript{44}, respectively, and van der Waals interactions between the methyl group on CDP-ME2P and the side chains of Ile\textsuperscript{57}, Leu\textsuperscript{60}, and Leu\textsuperscript{66}. The green dotted lines denote hydrogen and coordination bonds. The black arrows represent hypothetical electron flow during both the nucleophilic attack on the bridging phosphate and the breakdown of the putative pentacoordinate transition state releasing CMP and MECDP. (B) designates a second monomer.
likely catalytic mechanism accompanying cyclophosphosphate generation that is enzymatically controlled by proximity and metal cation-assisted catalysis (Fig. 3C). A key structural element in the proposed reaction mechanism is the four points binding of CDP-ME2P pictured in Fig. 3C. This arrangement precisely positions the bridging phosphate undergoing attack near the nonbridging phosphate that is the nucleophile responsible for the formation of the cyclophosphate moiety of MECDP. The tetragonally coordinated transition metal contributes to formation of the transition state accompanying cyclization by both arranging the phosphate undergoing nucleophilic attack and by serving to counterbalance the negative charge that develops in the pentacoordinate transition state (Fig. 3C).

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**REFERENCES**