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Solution NMR Structure, Backbone Dynamics, and Heme-Binding Properties of a Novel Cytochrome *c* Maturation Protein CcmE from *Desulfovibrio vulgaris*

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Supporting Information

ABSTRACT: Cytochrome *c* maturation protein E, CcmE, plays an integral role in the transfer of heme to apocytochrome *c* in many prokaryotes and some mitochondria. A novel subclass featuring a heme-binding cysteine has been identified in archaea and some bacteria. Here we describe the solution NMR structure, backbone dynamics, and heme binding properties of the soluble C-terminal domain of *Desulfovibrio vulgaris* CcmE, dvCcmE′. The structure adopts a conserved β-barrel OB fold followed by an unstructured C-terminal tail encompassing the CxxxY heme-binding motif. Heme binding analyses of wild-type and mutant dvCcmE′ demonstrate the absolute requirement of residue C127 for noncovalent heme binding in vitro.

c-Type cytochromes are small, ubiquitous heme proteins that employ a conserved CxxCH motif for binding a heme prosthetic group, where both cysteines covalently bind the vinyl groups of the heme via thiols and the histidine serves as the proximal axial ligand for the metal ion. The coordination sphere of the iron is generally completed by an axial methionine or histidine. Nature has evolved at least three different systems for the post-translational addition of heme to cytochrome *c*.[1−3] System I, relevant to this work, is generally comprised of eight cytochrome *c* maturation proteins (i.e., CcmABCDEFGH) and is prevalent in many prokaryotes and some protozoal and plant mitochondria. In this pathway, a small membrane-bound heme chaperone protein, CcmE, participates in the final transfer of heme to cytochrome *c*. CcmE is thought to form a transient covalent complex with heme through a conserved histidine (H130 in *Escherichia coli*) within a HxxxY motif near the C-terminus of the protein.[4,5] Structural studies of the soluble periplasmic C-terminal domain (lacking the N-terminal transmembrane helix) of *E. coli* and *Shewanella putrefaciens* apoCcmE revealed that the protein adopts a β-barrel fold immediately followed by the heme-binding motif and an unstructured C-terminal tail.[6,7] Although no holoCcmE structure has been reported to date, it has been established that binding of heme to *E. coli* CcmE occurs via a unique covalent bond between $N^{\delta 1}$ of H130 and a vinyl β-carbon within the heme,[8] the phenol moiety of Y134 serves as an axial ligand for the iron,[9,10] and the process occurs within a somewhat buried, hydrophobic heme-binding pocket.[11]

A System I variant subclass of CcmE proteins featuring a cysteine in place of the histidine in a CxxxY motif was predicted in archaea and some bacteria, including the sulfate-reducing *Desulfovibrio* species (Figure 1A).[12] It was recently demonstrated that placing the *Ccm* operon from *Desulfovibrio desulfuricans* into an *E. coli* strain lacking its endogenous Ccm system resulted in the production of holocytochrome *c*, and that cytochrome *c* maturation in this variant System I proceeds through the covalent attachment of heme to C127 in *D. desulfuricans* CcmE.[13]

Here, we present the solution NMR structure, backbone dynamics, and heme binding properties of the soluble C-terminal domain of *Desulfovibrio vulgaris* CcmE, dvCcmE′. On the basis of solution NMR structural and ^{15}N relaxation measurements, the apo form of dvCcmE′ adopts a β-barrel fold characteristic of the CcmE superfamily, featuring a highly dynamic C-terminal tail that includes the CxxxY heme-binding motif. In vitro heme binding studies of wild-type, C127A, and Y131F dvCcmE′ demonstrate that noncovalent ferric heme binding requires C127.

The construct optimization, cloning, expression, and purification of ^{13}C and ^{15}N isotopically enriched samples of *D. vulgaris* CcmE(44−128) lacking the nine C-terminal residues, and CcmE(44−137), dvCcmE′Δ9 and dvCcmE′, respectively (UniProtKB/TrEMBL entry Q72D78_DESVH; NESG entries DvR115G and DvR115, respectively) were

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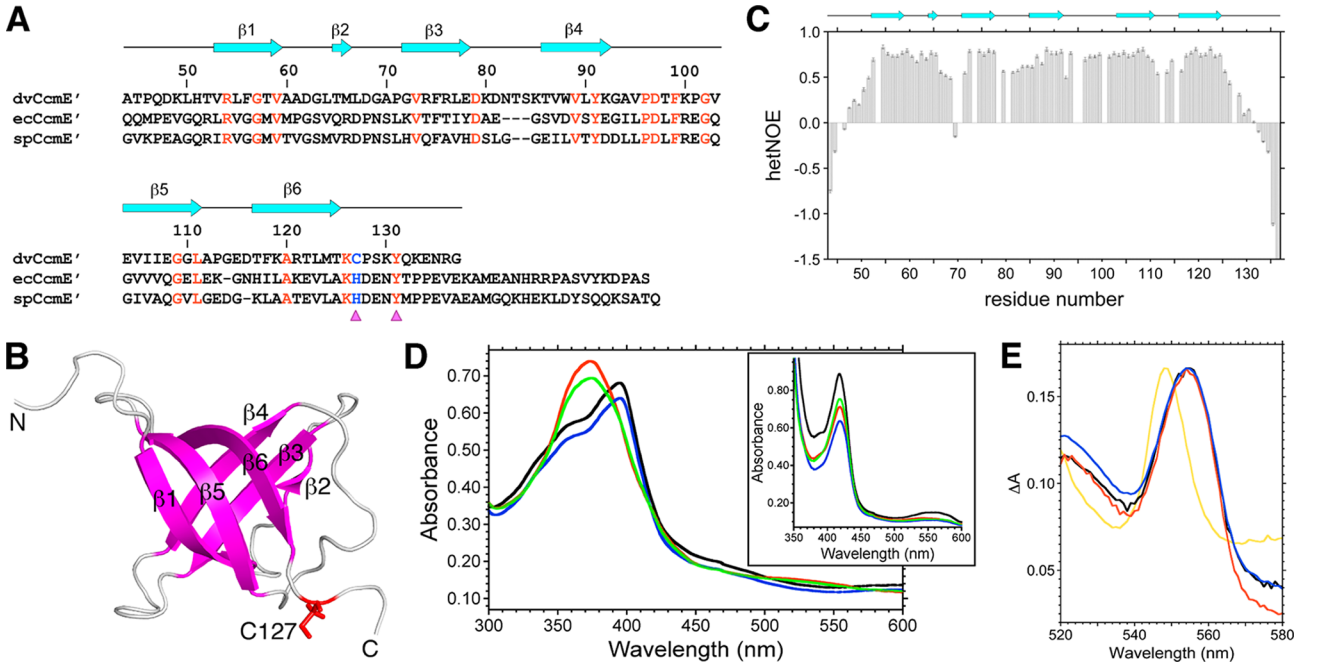

Figure 1. (A) Structure-based sequence alignment of the soluble C-terminal domains of *D. vulgaris* CcmE (dvCcmE′, residues 44−137), *E. coli* CcmE (ecCcmE′, residues 51−159), and *S. putrefaciens* CcmE (spCcmE′, residues 51−161). The sequence numbering and secondary structural elements for dvCcmE′ are shown above the alignment. Identical amino acid residues are colored red, and residues involved in heme binding are denoted below the alignment with magenta triangles. (B) Lowest-energy (CNS) conformer from the final solution NMR structure ensemble of apo-dvCcmE′Δ9. The β-strands and loops are colored magenta and gray, respectively. The heme-binding cysteine, C127, is colored red. Rendered using PyMOL (http://www.pymol.org). (C) Backbone dynamics of apo-dvCcmE′. Plot of ^{1}H−^{15}N heteronuclear NOE vs residue number obtained with [U-^{13}C,^{15}N]apo-dvCcmE′ at a ^{15}N Larmor frequency of 60.8 MHz. Secondary structural elements found in the solution NMR structure of dvCcmE′Δ9 are shown above the plot. (D) UV−visible absorption spectra of the ferric and ferrous (inset) forms of free heme (black), wild-type dvCcmE′ (red), C127A dvCcmE′ (blue), and Y131F dvCcmE′ (green). (E) Pyridine hemochrome spectra (reduced − oxidized) for free hemin (black), wild-type dvCcmE′ (red), C127A dvCcmE′ (blue), and equine heart holocytochrome *c* (gold). Only cyctochrome *c* exhibits the expected shift of the α-band diagnostic of covalent heme binding (λ_{max} = 549.5 nm).

conducted using standard protocols of the Northeast Structural Genomics Consortium[14] (see the Supporting Information for a complete description of the methods used in this work). Briefly, the solution NMR structure of apo-dvCcmE′Δ9 was determined at pH 4.5 by standard triple-resonance NMR methods using CYANA version 3.0,[15,16] followed by refinement in explicit water using CNS version 1.1.[17,18] A summary of constraints derived from the NMR data is presented in Table S1 of the Supporting Information, along with structure quality assessment statistics. The final ensemble of 20 models and constraints for dvCcmE′Δ9 were deposited in the Protein Data Bank (PDB) (entry 2KCT), and NMR resonance assignments, NOESY peak lists, ^{15}N−^{1}H residual dipolar couplings, and NOESY spectral FID data were deposited in the BioMagResBank (BMRB) (entry 16096). Residue-specific longitudinal and transverse relaxation rates (R_1 and R_2, respectively) and ^{1}H−^{15}N heteronuclear NOE values were obtained for apo-dvCcmE′ at pH 6.5 using standard two-dimensional gradient NMR experiments[19] (BMRB entry 18380). The in vitro heme binding properties of tagless wild-type, C127A, and Y131F dvCcmE′ were assayed by preparation of the ferric heme adduct,[20] followed by UV−visible absorption spectroscopy, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) with heme staining,[21] and pyridine hemochrome analysis;[22] reduced (ferrous) heme forms were produced by addition of sodium dithionite. The apo forms of both dvCcmE′Δ9 and dvCcmE′ are monomeric in solution under reducing conditions, based on gel filtration chromatography, static light scattering, and ^{15}N relaxation data. The expression plasmids for dvCcmE′Δ9 and dvCcmE′ can be accessed via the PSI Materials Repository (http://psimr.asu.edu/).

The solution structure of dvCcmE′Δ9 exhibits a classic β-barrel OB fold[23] comprised of six β-strands (β1, V53−V59; β2, T65−M66; β3, G72−E78; β4, T86−K92; β5, E104−L111; β6, T117−T125) arranged in a Greek key topology (Figure 1B). The functionally important cysteine, C127, is located just beyond the final strand. The structure of dvCcmE′Δ9 is remarkably similar to structures of the CcmE′ domains from *E. coli*[6] and *S. putrefaciens*,[7] in spite of the <30% sequence identity to the CcmE proteins from these organisms (Supporting Information). A conserved surface, ConSurf,[24] analysis of the cysteine-containing variant subfamily of the CcmE protein domain family reveals a high degree of amino acid residue conservation in the face of the OB fold comprising strands β1, β5, and β6, as well as the loop between strands β4 and β5 and the heme-binding C-terminus of the protein (Supporting Information). This conserved face of the molecule most likely participates in protein−protein interactions with CcmC.[25]

To probe the dynamics and heme binding properties of this variant CcmE, we designed tagless constructs of dvCcmE′ containing the complete native C-terminal region of the protein. ^{15}N NMR relaxation and ^{1}H−^{15}N heteronuclear NOE data (Figure 1C and Supporting Information) demonstrate that the ∼12 C-terminal residues immediately following the last β-strand in the structure, including the functionally important heme-binding region, are intrinsically disordered in

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apo-dvCcmE (^{1}H−^{15}N hetNOE of <0.5 for residues C127−G137; S^2 order parameter of <0.5 for residues S129−G137). In addition, several lines of experimental evidence conclusively establish that dvCcmE′ binds ferric heme in a noncovalent fashion but is incapable of covalent heme binding in vitro. In particular, UV−visible absorption spectroscopy (Figure 1D) and SDS−PAGE followed by heme staining (Supporting Information) demonstrate that C127 is absolutely required for binding of ferric heme to dvCcmE′ in vitro, whereas Y131 is not. Ferric heme adducts of wild-type and Y131F dvCcmE′ exhibit a dramatic Soret band shift (λ_{max} = 373 nm), whereas C127A dvCcmE′ emulates free hemin (λ_{max} = 396 nm). However, reduction of heme produces identical Soret band shifts for all species (Figure 1D, inset). Moreover, pyridine hemochrome spectra (Figure 1E), gel filtration chromatography, and mass spectrometry (not shown) further demonstrate that dvCcmE′ is incapable of covalently binding heme in vitro, in contrast to the related CcmE from *D. desulfuricans* that was shown to covalently bind heme via the conserved cysteine when produced in vivo.[13] The in vitro heme binding behavior of dvCcmE′ also contrasts with that reported for wild-type and H130C CcmE′ from *E. coli* that form a covalent bond to reduced heme after the initial noncovalent binding of ferric heme.[26,27] However, no evidence of heme binding was observed for the *S. putrefaciens* CcmE homologue.[7]

In summary, the solution NMR structure of the C-terminal heme-binding domain of *D. vulgaris* CcmE heme chaperone, dvCcmE′, reported here constitutes the first three-dimensional structure from the variant CcmE subfamily featuring a CxxxY heme-binding motif. We establish that the heme-binding motif in the apo form of dvCcmE′ is highly dynamic and intrinsically disordered in solution, and that residue C127 is required for noncovalent ferric heme binding in vitro. We postulate that covalent attachment of heme to this variant CcmE requires the intact (i.e., in vivo) Ccm machinery.

■ ASSOCIATED CONTENT

Supporting Information

Complete experimental methods, NMR and structural statistics for dvCcmE′Δ9 (Table S1), static light scattering data for dvCcmE′Δ9 (Figure S1), ^{15}N T_1 and T_2 relaxation data for dvCcmE′Δ9 (Figure S2), NMR sequential connectivity map for dvCcmE′Δ9 (Figure S3), superposition of the final ensemble of 20 conformers from the solution NMR structure of apo-dvCcmE′Δ9 (Figure S4), superposition of the solution structure of dvCcmE′Δ9 with that of *E. coli* CcmE′ (Figure S5), ConSurf analysis of the cysteine-containing CcmE subfamily (Figure S6), assigned ^{1}H−^{15}N HSQC spectrum of dvCcmE′ (Figure S7), complete ^{15}N relaxation analysis of dvCcmE′ (Figure S8), and heme-stained SDS−PAGE heme binding analysis of wild-type dvCcmE′ and its mutants (Figure S9). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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