Note

Expression, Purification, Physicochemical Characterization and Structural Analysis of Cytochrome c₅₅₄ from *Vibrio parahaemolyticus* Strain RIMD2210633

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The function of cytochrome c₅₅₄ of *Vibrio parahaemolyticus* has not yet been determined. We have determined the physicochemical properties and crystal structure of cytochrome c₅₅₄ at 1.8 Å in order to help elucidate its function. The physicochemical properties and the tertiary structure of cytochrome c₅₅₄ resemble those of dimeric cytochrome c₅₅₂ from *Pseudomonas nautica*, but the *Vibrio* genus contains no gene for nitrite reductase, cytochrome c₅₅₁, in its genome DNA. These results raise the possibility that both cytochromes denote an electron to an electron carrier and accept an electron from same electron carrier.

**Key words:** cytochrome c₅₅₄; crystal structure; *Vibrio parahaemolyticus*; physicochemical property

Bacterial electron transfer chains are composed of a variety of electron transport constituents, such as flavoproteins, iron sulfur proteins, quinones, and cytochromes, and the constituents of the system differ with the type of microorganism.¹,³ Soluble c-type cytochromes are ubiquitously distributed heme proteins, that act as electron carriers in the respiration of mitochondria and in photosynthetic electron transfer chains of chloroplasts.² The heme prosthetic group is covalently bound to the peptide chain, with the typical amino acid sequence Cys-X-X-Cys-His-consensus motif on the N-terminal. His and met residues are coordinated to heme Fe as axial ligands in the native form.

The *Vibrio* genus is composed of gram-negative facultatively anaerobic halophilic bacteria, and is the major causative agent of gastroenteritis. The complete genome sequences of *V. parahaemolyticus* strain RIMD2210633 have been published, and various metabolic processes have been predicted using this information.³ A large number of open reading frames containing this heme binding motif exist in *Vibrio* genome DNA, and some of them contain the gene for mono heme c-type cytochrome, which is annotated as cytochrome c₅₅₂, but the function of the cytochrome has not yet been determined. Based on sequence information on the *Vibrio* genome, this heme protein has one signal peptide, and is predicted to function as an electron carrier in the periplasmic space. We compared an amino acid sequence similarity of *Vibrio* cytochrome c₅₅₄ with *Pseudomonas nautica* cytochrome c₅₅₂, classified as a class I c-type cytochrome that forms a tight dimer, and it was 66% similarity. *Pseudomonas nautica* cytochrome c₅₅₂ functions as the electron donor to cytochrome c₅₅₁, the so-called nitrite reductase,⁴ but the *Vibrio* genus contains no gene for nitrite reductase cytochrome c₅₅₁ in its genome DNA, so the electron acceptor of *Vibrio* cytochrome c₅₅₄ is estimated to differ from *Pseudomonas* cytochrome c₅₅₂.

In this study, in order to compare physicochemical properties and tertiary structure as between *Vibrio* cytochrome c₅₅₄ and *Pseudomonas* cytochrome c₅₅₂, the cytochrome c₅₅₄ gene of *V. parahaemolyticus* strain RIMD2210633 (Vpc554) was expressed in *Escherichia coli*, the expressed recombinant protein was purified, and its physicochemical properties, UV/vis spectra and redox potential, were characterized. In addition, we determined the first crystal structure of Vpc554 at 1.8 Å resolution and compared it with that of *P. nautica* cytochrome c₅₅₂.

The gene encoding Vpc554 (VP2300, GenBank accession no. NC004603) was amplified from *V. parahaemolyticus* strain RIMD2210633 genome DNA using primers Vpc554-NdeI, 5'-ACCATATGAAAGGAGTAATGACGTGCC-3' (NdeI site underlined), and Vpc554-BamHI, 5'-AAAGGATCCCTATTCAACTTGAAGTAGTA-3' (BamHI site underlined). The product was cloned into the Ndel-BamHI sites of pET22b (+) (Novagen, Santa Clara, CA, USA) to construct plasmid pET22b-Vpc554. For overproduction of Vpc554, the expression vector was co-introduced with pSTV28ccmA-H into *E. coli* BL21 (DE3).⁵ Overexpression was performed using LB liquid medium or LB agar plates (per liter, 10 g of tryptone, 10 g of NaCl, 5 g of yeast extract, pH 7.0) containing 100 µg/ml of ampicillin and 25 µg/ml of chloramphenicol. Freshly plated colonies were used to inoculate 1.5 liter of LB liquid medium in a 3.0-liter flask, followed by incubation for 36 h at 100 rpm at 30 °C. Cells were harvested by centrifugation at 6,000 g (4 °C) for 5 min. The pellet was resuspended in 80 ml of PBS buffer and disrupted.

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with a high-pressure homogenizer (Mini Lab 8.30H, Albertlund, Denmark). The suspension was fractionated with ammonium sulfate (30–70% saturation). The precipitate was dissolved in a small amount of 20 mM sodium acetate buffer (pH 4.5) and dialyzed against the same buffer. The sample was applied to a CM52 cellulose column (Whatman, the same buffer. The sample was applied to a Dowex 50W-X8 column (Bio-Rad, the same buffer). The flowthrough was collected and concentrated by ultrafiltration using a VM500 column (Millipore, the same buffer). The sample was then applied to a CM52 cellulose column (Whatman, the same buffer) and eluted with 20 mM sodium acetate buffer (pH 4.5). The UV/visible spectra of the recombinant Vpc554 were measured following overnight storage of the sample.

The crystal structure of Vpc554 was determined at 1.8 Å resolution. This is the first cytochrome c554 crystal structure from the Vibrio genus. The crystal belonged to space group P1 with unit cell parameters a = 84.95, b = 87.61, c = 103.85 Å, and the asymmetric unit contained 16 protomer dimers. These 32 molecules were superimposed, with main-chain root-mean-square deviation (RMSD) values of 0.2–1.1 Å, determined using the DALI program.

The monomer Vpc554 proved to belong to class I c-type cytochromes, which are composed of a single polypeptide chain folded around the c-type heme prosthetic group. The secondary structures were classified according to the criteria of Kabsch and Sander. Four α-helices, Ala3-His17 (I), Glu36-Asn48 (II), Ala57-Ala63 (III), and Asp68-Ser80 (IV), are found as α-helical barrels. The Gly18-Asn35 region followed by Ala3-His17 forms an 8-strand β-sheet and where stereochemically reasonable hydrogen bonds were allowed. A summary of data collection and refinement statistics is given in Table 1. The refined crystallographic coordinates and structure factor amplitudes have been deposited in the Protein Data Bank (PDB ID code 2ZZS).

The crystal structure of Vpc554 was determined by molecular replacement using the program MOLREP.9) The search model used was P. nautica cytochrome c552.4) The structure of Vpc554 was refined with Refmac using the CCP4 program suite. Water molecules were added using a water pick script of CNS, and refinement was continued using REFMAC8. The final model obtained had an R-factor of 19.2% and a free R-factor of 24.2%. Manual model building was carried out using Coot.10) Solvent molecules were placed at positions where spherical electron density peaks were found above 1.5σ in the [2Fo − Fo] map and above 3.0σ in the [Fo − Fo] map, and where stereochemically reasonable hydrogen bonds were allowed. A summary of data collection and refinement statistics is given in Table 1. The refined crystallographic coordinates and structure factor amplitudes have been deposited in the Protein Data Bank (PDB ID code 2ZZS).

For crystallization of Vpc554, the purified recombinant Vpc554 was dissolved in super-pure water to prepare a concentrated protein solution of 20 mg ml⁻¹. Vpc554 was crystallized by vapor diffusion; the hanging drops used contained a 1:1 mixture of protein and reservoir solution. The Vpc554 was allowed to crystallize over a reservoir containing 0.1 M phosphate-citrate (pH 4.2), 0.2 M NaCl, and 20% (w/v) PEG-8000. X-Ray diffraction data were collected on a BL-5A (Photon Factory, Tsukuba, Japan). The data set was processed with HKL2000 and scaled with SCALEPACK.8) The structure of Vpc554 was determined by molecular replacement using the program MOLREP.9) The search model used was P. nautica cytochrome c552.4) The structure of Vpc554 was refined with Refmac using the CCP4 program suite. Water molecules were added using a water pick script of CNS, and refinement was continued using REFMAC8. The final model obtained had an R-factor of 19.2% and a free R-factor of 24.2%. Manual model building was carried out using Coot.10) Solvent molecules were placed at positions where spherical electron density peaks were found above 1.5σ in the [2Fo − Fo] map and above 3.0σ in the [Fo − Fo] map, and where stereochemically reasonable hydrogen bonds were allowed. A summary of data collection and refinement statistics is given in Table 1. The refined crystallographic coordinates and structure factor amplitudes have been deposited in the Protein Data Bank (PDB ID code 2ZZS).

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The monomer Vpc554 proved to belong to class I c-type cytochromes, which are composed of four α-helices and tight turns (Fig. 2A). This protein consists of a single polypeptide chain folded around the c-type heme prosthetic group. The secondary structures were classified according to the criteria of Kabsch and Sander. Four α-helices, Ala3-His17 (I), Glu36-Asn48 (II), Ala57-Ala63 (III), and Asp68-Ser80 (IV), are found as α-helical barrels. The Gly18-Asn35 region followed by Ala3-His17 forms an 8-strand β-sheet and where stereochemically reasonable hydrogen bonds were allowed. A summary of data collection and refinement statistics is given in Table 1. The refined crystallographic coordinates and structure factor amplitudes have been deposited in the Protein Data Bank (PDB ID code 2ZZS).

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R.m.s.d. bond length/bond angle (Å) 0.018/1.777
cut off/reflections used 0.0/235782
Resolution range (Å) 20–1.80

Refinement statistics

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<td>Residues in most favorable region (%)</td>
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Table 1. Crystal Parameters, Data Collection, and Structure Refinement

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<td>Unit cell parameters (Å)</td>
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<tr>
<td></td>
<td>α = 71.50°, β = 71.50°, γ = 83.68°</td>
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<tr>
<td>Reflections (Measured/Unique)</td>
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<td>Completeness (Overall/Outer Shell, %)</td>
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<td>Rmerge (Overall/Outer Shell, %)</td>
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<tr>
<td>Redundancy (Overall)</td>
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<tr>
<td>Mean [I/Ij]</td>
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</table>

Refinement factors were calculated with 5% of the data excluded from the refinement. Values in parentheses are for the outer shell, with resolution within 20.0–1.80 Å.

this dimer conformation resembles dimeric cytochrome c552 from P. nautica. A comparison between the amino acid sequences of Vpc554 and P. nautica cytochrome c552 showed 66% similarity. The main-chain RMSD value between Vpc554 and Pseudomonas cytochrome c552 was 0.6 Å, and a Cα trace of Vpc554 showed high overall similarity with P. nautica cytochrome c552 (Fig. 2B). The α-helices and Ω-shaped loop of Vpc554 are also observed at the corresponding positions in P. nautica cytochrome c552. The similarity of the amino acids at the dimer interface of the two cytochromes is not much higher than that of other regions, but the hydrogen bond network and electrostatic interaction around two heme propionates at the dimer interface of the two proteins are very similar (Fig. 2C). Two Arg51s of the dimer form electrostatic interactions with the heme propionates, and the negative charges of the heme propionates are neutralized by these two positively charged amino acid residues. This electrostatic interaction of Vpc554 might contribute to the rigid packing of the interaction between monomers.

The biological function of Vpc554 remains obscure. The Pseudomonas genus contains the gene for nitrite reductase, cytochrome cd1, in its genome. On the other hand, the Vibrio genus contains no gene for cytochrome cd1 in its genome, so Vpc554 and P. nautica cytochrome c552 might donate electrons to different electron carriers. But, considering that the redox potential and the tertiary structure of Vpc554 are similar to those of P. nautica cytochrome c552, the two cytochromes are predicted to accept an electron from the same electron carrier.

Acknowledgment

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References


Fig. 2. (A) Ribbon Representation of the Secondary Structure of Vpc554, (B) Superimposition of Co Traces, and (C) Environment around the Two Heme Propionates at the Dimer Interface of Vpc554 (Blue) and Pseudomonas nautica Cytochrome c552 (Red).