Comparative Analysis of Highly Homologous Shewanella Cytochromes c$_5$ for Stability and Function

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Homologous cytochromes c$_5$ from a mesophile, Shewanella amazonensis (SA cyt$_c$), and a psychrophile, Shewanella violacea (SV cyt$_c$), were compared to elucidate the molecular mechanisms underlying protein stability and function. Cyclic voltammetry revealed that the two proteins had the same redox potential value. Differential scanning calorimetry showed that SV cyt$_c$ was more stable than SA cyt$_c$ in an enthalpic manner. These results and the structure model of Shewanella oneidensis cytochrome c$_5$ indicated that hydrophobic heme environments in the two proteins are the same to maintain the same redox potential value, and that the intra-molecular interactions in SV cyt$_c$ are similar to those in SA cyt$_c$.

Key words: cytochrome c; electron transfer; protein stability; redox potential; Shewanella

Clues to the relationships among protein structure, stability, and function can be obtained by pairwise comparison of homologous proteins from extremophiles and organisms living under normal conditions. Cytochromes c, isolated from a variety of organisms, are useful in investigations of the structural basis of protein stability and function at the amino-acid level. In this context, we have carried out comparative analyses using four homologous mono-heme cytochromes c from mesophilic Pseudomonas aeruginosa,2) thermophilic Hydrogenophilus thermoluteolus3) and Hyperthermophilus thermophilus,5) and hyperthermophilic Aquifex aeolicus.5)

Genus Shewanella belongs to the Gamma-proteobacteria, and is distributed worldwide in ocean areas from the deep to shallower.5) Shewanella is also characterized by having mono-heme cytochromes c$_5$, which are classified in Ambler’s Class IE cytochrome c,7) and they occupy close positions in the phylogenetic tree (Fig. 1A). Shewanella cytochromes c$_5$ contain two conserved Cys residues (Cys-59 and Cys-62, Fig. 1B) that form an intra-molecular disulfide bond, as exemplified in Shewanella oneidensis cytochrome c$_5$ (SO cyt$_c$) (Fig. 1C).5) We have found that the disulfide bond in cytochrome c$_5$ (SV cyt$_c$) of psychophilic Shewanella violacea, isolated from deep sea sediments growing optimally at 8°C, affects protein stability but not the redox potential value.5)

In order to investigate further the molecular mechanisms underlying protein stability and function at the amino-acid level, here we compared SV cyt$_c$ and its mesophilic counterpart, cytochrome cyt$_c$ (SA cyt$_c$) of Shewanella amazonensis, isolated from the Amazon River Delta, growing optimally at 35–37°C.10) Although the two bacteria, S. violacea and S. amazonensis, are of course adapted to different temperature environments, the two cytochromes c, SV cyt$_c$ and SA cyt$_c$, have exactly the same amino acid numbers, unlike another mesophilic structure-determined SO cyt$_c$, which has two extra residues (Fig. 1B). Therefore SV cyt$_c$ and SA cyt$_c$ are excellent experimental models to obtain clues to protein stability and function without mutagenesis study.

Materials and Methods

Cloning of the SA cyt$_c$ gene. Based on the genome sequence of S. amazonensis, we designed PCR primers to amplify the gene encoding SA cyt$_c$ including its signal sequence, from chromosomal DNA. The SA cyt$_c$ gene was inserted into the pKK223-3 vector (ampicillin resistance) under the control of the tac promoter, which was then used in the expression of the SA cyt$_c$ gene in Escherichia coli.15)

Protein preparation. The SV cyt$_c$ and SA cyt$_c$ proteins were prepared by a method previously described.19) The proteins were overexpressed with co-transformed pEC86 carrying the ccmABC-DEFGH genes in E. coli JCB387 cells and, were isolated from the periplasmic extracts. They were first purified by DEAE Toyopearl (Tosoh, Tokyo) column chromatography, followed by Hi-Trap Q and SP (GE Healthcare, Buckinghamshire), as described previously.19)

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Abbreviations: CV, cyclic voltammetry; DSC, differential scanning calorimetry; SA cyt$_c$, Shewanella amazonensis cytochrome c$_5$; SO cyt$_c$, Shewanella oneidensis cytochrome c$_5$; SV cyt$_c$, Shewanella violacea cytochrome c$_5$
Fig. 1. Sequence Analysis of Mono-Heme Cytochromes c.

A. Phylogenetic tree of mono-heme cytochromes c. Multiple-sequence alignment was carried out using ClustalW. Tree topology and evolutionary distance estimations were performed by the neighbor-joining method. Bar, 0.5 substitutions per amino acid positions. B. Sequence alignment of SV cytC, SA cytC, and SO cytC. Identical and variable residues in SV cytC and SA cytC are shown by black and gray bold letters respectively. SO cytC residues are represented by plain letters, and side-chain atoms of underlined residues are located within 3 Å of heme atoms up to residue no. 76. C. Three-dimensional structure of SO cytC (PDB code, 1KX2). The heme and relevant amino acid residues are drawn as a stick model.

Finally, gel-filtration chromatography was performed on a Superdex 75 column (GE Healthcare) equilibrated and eluted with 10 mM Tris–HCl buffer pH 8.0 at 4 °C. The purified SA cytC protein was subjected directly to N-terminal sequencing with an automatic peptide sequencer (Applied Biosystems, Tokyo). The amino acid concentrations were estimated by the Bradford method using bovine serum albumin as a standard.11) Judging by the amino acid sequences of the mature parts of the proteins without heme, the molecular mass values are 8002 and 8029 respectively. In both proteins the disulfide bond between Cys-59 and Cys-62 might have formed during the purification procedure and data analysis were as described previously.12) The resulting suspensions were centrifuged (200,000 × g for 1 h, and then 220,000 g for 1.3 h), and the pellets were resuspended with membrane wash buffer (50 mM Tris–HCl, 2 mM MgCl₂, 1 mM DTT, 10% glycerol, pH 8.0, 4 °C). The resulting suspensions were used as membrane fractions in the cytochrome c oxidase activity assay.

Cyclic voltammetry. Methods of cyclic voltammetry (CV) were applied to the purified SV cytC and SA cytC proteins in order to determine their midpoint redox potential (E₅₀) values relative to the standard hydrogen electrode (SHE) at 25 °C at pH 5.0. The detailed experimental procedure and data analysis were as described previously.12)

Protein denaturation. Thermal denaturation experiments were performed by differential scanning calorimetry (DSC). The solutions of air-oxidized SV cytC and SA cytC proteins were diazlyzed extensively against 50 mM sodium acetate buffer (pH 4.0 and 3.6) before the measurements. Under these pH conditions, thermal denaturation of the SV cytC and SA cytC proteins was observed in a reversible manner, providing equilibrium thermodynamic parameters. Hence, these pHs were used to measure thermal stability. The degassed protein solutions (about 60 μM) were then loaded into a calorimeter cell and heated from 10 to 120 °C at about 28 ps, at a heating rate of 1 °C min⁻¹, with a calorimeter VP-DSC (Microcal, Piscataway). Buffer-buffer base lines were recorded at the same heating rate and then subtracted from the sample curves to obtain heat capacity (Cᵥ) curves. After fitting the data with MATHEMATICA 7.0, the transition temperature during thermal denaturation (Tₘ) and the calorimetric enthalpy change (ΔHₘ) at Tₘ were obtained.

The heat capacity change accompanied by the thermal denaturation, ΔCᵥ, was estimated as a function of temperature, as described previously.9,13) From these values, thermodynamic parameters (free energy change ΔG, enthalpy change ΔH, and entropy change ΔS) at a given temperature were calculated using the following equations:

\[ ΔH(T) = ΔH_₀ + ΔCᵥ(T)(Tₘ - T) \]
\[ ΔS(T) = ΔH(T)/Tₘ - ΔCᵥ(T) ln(Tₘ/T) \]
\[ ΔG(T) = ΔH(T) - TΔS(T) \]

These calculations facilitated comparison of thermodynamic protein stability, as described previously.9,13)

Membrane preparation. Cells of S. violacea and S. amazonensis (10 g wet weight) were harvested from marine broth (at 8 °C) and LB (at 37 °C) cultures respectively, and resuspended in 70 mL of TKG buffer containing 20 mM Tri-HCl (pH 8.0, 4 °C), 140 mM KCl, and 10% v/v glycerol with 1 μM phenylmethylsulfonyl fluoride in 99% EtOH, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, and 1 μg/ml DNase I. The cells were then disrupted with a French pressure cell. Unbroken cells were removed by centrifugation (15,000 × g for 10 min 3 times). The resulting supernatants were centrifuged (200,000 × g for 1 h, and then 220,000 g for 1.3 h), and the pellets were resuspended with membrane wash buffer (50 mM Tris–HCl, 2 mM MgCl₂, 1 mM DTT, 10% glycerol, pH 8.0, 4 °C). The resulting suspensions were used as membrane fractions in the cytochrome c oxidase activity assay.

Cytochrome c oxidase activity assay. A cytochrome c oxidase activity assay of the membrane fractions was performed spectrophotometrically. The reaction mixture contained dithionite-reduced purified SV cytC or SA cytC proteins (about 20 μM) and freshly prepared membrane fractions from S. violacea or S. amazonensis cells (40 μg of protein) in a total volume of 0.4 ml 10 mM HEPES buffer (pH 7.0). The reactions were initiated by the addition of membrane fractions. Oxidation of the reduced cytochromes c was monitored at 20 °C as the decrease in absorbance at 553 nm, and initial rates were calculated using a molar coefficient for both proteins at 553 nm of 24 mM⁻¹ cm⁻¹. Cytochrome c oxidase activity was then estimated from the initial rate of the intact membrane fraction by subtracting that obtained from the mixture without the membrane fraction which, we assumed, gave non-enzymatic activity. Specific activity was expressed in nmoles of cytochrome c oxidized per min per mg of membrane protein. In order to verify the cytochrome c oxidase activity of the bacterial membrane proteins, horse heart cytochrome c was also tested as an electron donor.

Results

Expression of SA cytC in the E. coli periplasm

The N-terminal amino acid sequence of the recombinant SA cytC expressed in the E. coli periplasm was determined to be Gln-Glu-Gly-Glu-Ala, identical to the putative sequence for the mature protein (Fig. 1B). Thus, the original SA cytC signal peptide was correctly
E. coli e

cent heat capacity changes (capacity (C)), experiments were carried out with CV. The thermal denaturation. Using the

SV cyt5, the protein stability. At these pH values, the values for pH values, both giving equilibrium thermodynamic

denaturation of SV cyt5, analyses.

E. coli cyt5, were examined in subsequent analyses.

Redox potential analysis

In order to determine whether the difference in protein sequence between SV cyt5 and SA cyt5 would correlate with the redox potential (Em) value, electrochemical experiments were carried out with CV. The Em values for the SV cyt5 and SA cyt5 proteins were +326.7 ± 0.5 and +326.2 ± 0.5 mV versus a standard hydrogen electrode, respectively. These values were the same within the limits of significance.

Thermal denaturation as monitored by DSC

Excess molar heat capacity curves for the air-oxidized SV cyt5 and SA cyt5 proteins were obtained through DSC measurements at pH 4.0 and 3.6 (Fig. 2). The two pH values, both giving equilibrium thermodynamic parameters, were employed to determine pH effects on the protein stability. At these pH values, the values for the transition temperature during thermal denaturation (Tm) were larger in SV cyt5 than in SA cyt5 (Table 1), indicating the higher stability of the former.

From the curves for the observed excess molar heat capacity (Cp, Fig. 2), we obtained temperature-dependent heat capacity changes (ΔCp) accompanied by the thermal denaturation. Using the ΔCp value at the Tm of the SA cyt5 protein (79.8 °C, Tm5 at pH 4.0 and 73.9 °C at pH 3.6), ΔCp(Tm5), other thermodynamic parameters at 79.8 and 73.9 °C, ΔG(Tm5), ΔH(Tm5), and ΔS(Tm5), would be compared for the SV cyt5 and SA cyt5 proteins (Table 1). Since the Tm value is defined to be equivalent to the temperature at which ΔG becomes zero, the ΔG(Tm5) value of the SA cyt5 protein was zero. The SV cyt5 ΔG(Tm5) values at pH 4.0 and 3.6 were 4.24 and 5.34 kJ mol⁻¹, respectively, these being positive as compared with those of the SA cyt5 protein at the pH values tested, indicating that the stability of SV cyt5 is thermodynamically higher.

The ΔG value can be dissected into enthalpy (ΔH) and entropy (ΔS) terms, as shown by the equation ΔG = ΔH − TΔS. At pH 4.0, the SV cyt5 ΔS(Tm5) value (0.902 kJ mol⁻¹ K⁻¹) was equal to that of the SA cyt5 protein (Table 1), indicating that the two proteins have thermodynamically the same features in an entropic term. However, the SV cyt5 ΔH(Tm5) value (322.6 kJ mol⁻¹) at pH 4.0 was significantly larger than that of the SA cyt5 protein (318.3 kJ mol⁻¹), contributing to the large ΔG(Tm5) value of SV cyt5 as compared with that of SA cyt5 (Table 1). Therefore, the thermal stability of SV cyt5 is due only to the enthalpic effect as compared with SA cyt5 at pH 4.0.

At pH 3.6, the SV cyt5 ΔS(Tm5) value (0.844 kJ mol⁻¹ K⁻¹) was 1.8% larger than that of the SA cyt5 protein (0.829 kJ mol⁻¹ K⁻¹), indicating that SV cyt5 is unfavorable in an entropic term (Table 1). However, the SV cyt5 ΔH(Tm5) value (297.9 kJ mol⁻¹) at pH 4.0 was 3.6% larger than that of the SA cyt5 protein (287.3 kJ mol⁻¹), contributing to the large ΔG(Tm5) value of SV cyt5 as compared with that of the SA cyt5 (Table 1). Therefore, the thermal stability of SV cyt5 was due to the enthalpic effect, overcoming an accompanying unfavorable entropic effect as compared with SA cyt5 at pH 3.6. Although the entropic effect on the stability of the SV cyt5 and SA cyt5 proteins differs at pH 4.0 and 3.6, we can conclude that SV cyt5 is more stable than SA cyt5 in an enthalpic manner at both pH values.

Cytochrome c oxidase activity assay

The reduced SV cyt5 and SA cyt5 proteins were oxidized in the presence of S. violaceae and S. amazonensis membranes fractions (Fig. 3), indicating that both membrane fractions have cytochrome c oxidase activity with these cytochromes c as electron donors. Both membrane fractions also had oxidase activity for an artificial electron donor, horse heart cytochrome c (25.3 nmol of cytochrome c oxidized per min per mg of membrane protein from S. amazonensis), indicating that the electron transfer reaction involved in cytochrome c is conserved in these bacteria and mammalian mitochondria. Comparing the oxidation rates of reduced SV cyt5 and SA cyt5 proteins, it was obvious that the rates of SV cyt5 with the both membranes were faster than those of SA cyt5 with the same membranes (Fig. 3).

Discussion

The aim of this study was to compare the highly homologous SV cyt5 and SA cyt5 proteins in order to predict the molecular mechanisms underlying stability and function at the amino-acid residue level without
performing a mutagenesis study. From the three-dimen-
sional structure model of SO cyt c, having 77% amino
acid sequence identity to SV cyt c and 81% to SA cyt c,
one can predict the intra- and inter-molecular interac-
tions that caused the present experimental observations.

Our CV analysis showed that SV cyt c and SA cyt c
had the same redox potential value. Nineteen residues
(the underlined ones in Fig. 1B) of SO cyt c formed
close hydrophobic contact with the heme group (within 3 Å),
which are supposed to be responsible for regulat-
ing redox potential value, as suggested previously. Among
these, 17 residues are identical within the sequences of
SV cyt c and SA cyt c (Fig. 1B), indicating that the heme
environments are similar in these two proteins, resulting in
the same redox potential value. Among the 19 residues,
amino acid substitutions between the SV cyt c and SA cyt c proteins occurred
at the corresponding positions of Val-13 and Leu-50 in
SO cyt c (Fig. 1B, C). These might compensate
hydrophobic heme environments in SV cyt c and SA cyt c
with each other to maintain the same redox potential
value.

It sounds discrepant that psychrophilic S. violacea has
more stabilized SV cyt c than mesophilic S. amazonen-
sis SA cyt c, as revealed by the present DSC analysis.
However, considering that S. violacea is also charac-
terized as a piezophile, it is possible that SV cyt c is
stabilized against pressure as well as heat as compared
with SA cyt c. Observations supporting this idea are also
available for several highly homologous glyceralde-
hydes-3-phosphate dehydrogenases from mesophilic
and thermophilic sources.

Although the correlation between thermal and pres-
sure stabilization in the SV cyt c and SA cyt c proteins

is still unclear, a possible molecular mechanism under-
lying the thermal stability difference can be predicted
from the structure model of SO cyt c (Fig. 1C). One of
the candidate responsible residues is Tyr-73 in SV cyt c.
The amino acid replacement at this position causes a different
interaction between the N- and C-terminal helices.
Furthermore, its side chain is located away from the
heme (by over 6 Å), thus not affecting the redox
potential value. In addition, SV cyt c Lys-50 perhaps
contributes to higher stability, because it can form an ion
pair with the heme propionic group with the aid of Gly-
51, forming a void space intruding on the Lys-50 side
chain near the heme group. The corresponding residue
in SA cyt c is Leu-50, which cannot form an ion pair,
as shown in the SO cyt c structure (Fig. 1C).

Since the SV cyt c and SA cyt c proteins have the
same redox potential values, the difference in the
electron transfer rate from cytochromes c to the
membrane proteins is due to inter-molecular interac-
tions, not to intra-molecular interactions, which should
affect the redox potential. One of the residues perhaps
responsible for this difference is Lys-4 in SV cyt c,
which is replaced with Glu-4 in SA cyt c. This is
because the corresponding residue in SO cyt c is located
on the outer surface of the molecule (Fig. 4) and the
region containing this residue is near the hydrophobic
patch responsible for contact with various redox part-
ers, as discussed previously. Solvent-exposed Lys-4 in SV cyt c might efficiently regulate fast

### Table 1. $T_m$ Values and Thermodynamic Parameters at $T_m$ of SA cyt c ($T_m^*$) Obtained by DSC

<table>
<thead>
<tr>
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<th>$T_m$ (°C)</th>
<th>$\Delta H(T_m^*)$ (kJ mol$^{-1}$)</th>
<th>$\Delta S(T_m^*)$ (kJ mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta G(T_m^*)$ (kJ mol$^{-1}$)</th>
<th>$\Delta C_p(T_m^*)$ (J mol$^{-1}$ K$^{-1}$)</th>
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<tbody>
<tr>
<td>pH 4.0</td>
<td></td>
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<tr>
<td>SV cyt c</td>
<td>84.5 ± 0</td>
<td>322.6 ± 1.0</td>
<td>0.902 ± 0.003</td>
<td>4.24 ± 0.02</td>
<td>1.82 ± 0.56</td>
</tr>
<tr>
<td>SA cyt c</td>
<td>79.8 ± 0</td>
<td>318.3 ± 0.4</td>
<td>0.902 ± 0.001</td>
<td>0</td>
<td>2.04 ± 1.07</td>
</tr>
<tr>
<td>pH 3.6</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SV cyt c</td>
<td>80.1 ± 0</td>
<td>297.9 ± 0.5</td>
<td>0.844 ± 0.001</td>
<td>5.34 ± 0.03</td>
<td>4.50 ± 0.28</td>
</tr>
<tr>
<td>SA cyt c</td>
<td>73.9 ± 0.1</td>
<td>287.3 ± 1.4</td>
<td>0.829 ± 0.004</td>
<td>0</td>
<td>3.19 ± 1.15</td>
</tr>
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$T_m^*$ represents the $T_m$ of SA cyt c (79.8°C at pH 4.0 and 73.9°C at pH 3.6) as monitored by DSC. $\Delta H(T_m^*)$, $\Delta S(T_m^*)$, $\Delta G(T_m^*)$, and $\Delta C_p(T_m^*)$ are the values at $T_m^*$. Errors are estimated for at least three independent measurements.

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**Fig. 3.** Cytochrome c Oxidase Activity Assay.
Reduced SV cyt c and SA cyt c were oxidized in the presence of membrane proteins from S. violacea (V) and S. amazonensis (A).

**Fig. 4.** Surface Models of SV cyt c and SA cyt c.
Surface representations of SV cyt c and SA cyt c are based on the SO cyt c structure model (PDB code: 1KX2) and the substituted residues in SV cyt c and SA cyt c are introduced in silico. Side chains, which have positive or negative full charges, are colored in blue and red. These figures were prepared using the default values for electrostatic potential calculations of the program PyMOL.
association and dissociation between the cytochrome c and its redox partner. This ought to be proved using purified enzymes performing this electron transfer reaction in the future.

Here we found experimentally that SV cyt\textsubscript{c5} and SA cyt\textsubscript{c5} have the same redox potential value, while these two proteins differ in stability against thermal denaturation and rate of electron transfer to membrane proteins. The above predictions for intra- and inter-molecular interactions in the SV cyt\textsubscript{c5} and SA cyt\textsubscript{c5} proteins are based on their high sequence identity and a structural model of SO cyt\textsubscript{c5}. These materials should enable one to elucidate the molecular mechanisms underlying protein stability and function without mutagenesis study.

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References