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Stabilization of *Pseudomonas aeruginosa* Cytochrome c_{551} by Systematic Amino Acid Substitutions Based on the Structure of Thermophilic *Hydrogenobacter thermophilus* Cytochrome c_{552}*

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A heterologous overexpression system for mesophilic *Pseudomonas aeruginosa* holocytochrome c_{551} (PA c_{551}) was established using *Escherichia coli* as a host organism. Amino acid residues were systematically substituted in three regions of PA c_{551} with the corresponding residues from thermophilic *Hydrogenobacter thermophilus* cytochrome c_{552} (HT c_{552}), which has similar main chain folding to PA c_{551}, but is more stable to heat. Thermodynamic properties of PA c_{551} with one of three single mutations (Phe-7 to Ala, Phe-34 to Tyr, or Val-78 to Ile) showed that these mutants had increased thermostability compared with that of the wild-type. Ala-7 and Ile-78 may contribute to the thermostability by tighter hydrophobic packing, which is indicated by the three dimensional structure comparison of PA c_{551} with HT c_{552}. In the Phe-34 to Tyr mutant, the hydroxyl group of the Tyr residue and the guanidyl base of Arg-47 formed a hydrogen bond, which did not exist between the corresponding residues in HT c_{552}. We also found that stability of mutant proteins to denaturation by guanidine hydrochloride correlated with that against the thermal denaturation. These results and others described here suggest that significant stabilization of PA c_{551} can be achieved through a few amino acid substitutions determined by molecular modeling with reference to the structure of HT c_{552}. The higher stability of HT c_{552} may in part be attributed to some of these substitutions.

Proteins isolated from thermophilic organisms are usually stable to heat, indicating that these proteins must themselves embody most of the determinants of protein thermostability. Comparative studies of homologous proteins from mesophiles and thermophiles have provided ideas to explain elevated thermostability which include relatively small solvent-exposed surface area (1), increased packing density (2–4) and core hydrophobicity (5, 6), decreased length of surface loops (4), and generations of ion pairs or hydrogen bonds between polar residues (7, 8). Some recent site-directed mutagenesis studies have indicated that significant stabilization occurs in proteins as a result of mutations to reduce the entropy of the unfolded state (9, 10).

Cytochrome *c* is characterized by covalent attachment of the heme to the polypeptide chain. This protein has proved useful as a model system for studying the relationship between protein structure and stability because (i) primary and three-dimensional structures of cytochromes *c* from a wide variety of organisms (both mesophiles and thermophiles) are available, and (ii) heterologous expression systems of both prokaryotic and eukaryotic holocytochromes *c* have been established (11, 12), which facilitate site-directed mutagenesis studies.

Cytochrome c_{552} (HT c_{552})[1] from a thermophilic hydrogen oxidizing bacterium, *Hydrogenobacter thermophilus* that grows optimally at 70 °C, is an 80-amino acid protein with a heme. HT c_{552} has 56% sequence identity to an 82-amino acid monoheme cytochrome c_{551} (PA c_{551}) from mesophilic *Pseudomonas aeruginosa* (13), and the main chain foldings of these proteins are almost the same (14). As expected from the optimal growth temperatures of *H. thermophilus* and *P. aeruginosa*, HT c_{552} is more stable to heat than PA c_{551} (15). The genes encoding both proteins have been cloned (16, 17) with a view to identifying (by site-directed mutagenesis) amino acid residues that contribute to the higher stability of HT c_{552} compared with PA c_{551}. PA c_{551} and HT c_{552} are thus very suitable proteins for identifying substitutions of amino acid residues that endow stability.

Here we report that holo-PA c_{551}, which in terms of visible absorption spectra and thermostability is indistinguishable from the native protein, could be expressed in the periplasm of *Escherichia coli*. Using this expression system, site-directed mutagenesis studies were performed to show that the stability of PA c_{551} could be significantly increased through selected mutations, which had been chosen by molecular modeling with reference to corresponding amino acid residues in HT c_{552}. We discuss the structural origins of higher stability of HT c_{552}.

EXPERIMENTAL PROCEDURES

Bacterial Strain, Plasmids, and Growth Condition—The *Eco*RI-*Pst*I gene fragment *CP1*, encoding the 22-amino acid signal sequence and

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[1] The abbreviations used are: HT c_{552}, ferrocytochrome c_{552} from *H. thermophilus*; GdnHCl, guanidine hydrochloride; PCR, polymerase chain reaction; PA c_{551}, ferrocytochrome c_{551} from *P. aeruginosa*; HP c_{552}, ferrocytochrome c_{552} from *H. thermoluteolus*; CD, circular dichroism; HSQC, heteronuclear single quantum correlation.

82-amino acid mature protein of PA c_{551} (18), was inserted into the corresponding restriction sites of pKK223–3 (Amersham Pharmacia Biotech) to generate pKPA1. The pKPA1-based plasmids carrying mutated PA c_{551} gene fragments (see below) were transformed by standard methods into *E. coli* JCB7120 strain in which the expression of *c*-type cytochromes is unusually high.[2] The transformed *E. coli* cells were grown anaerobically in minimal media in the presence of glycerol, nitrite, and fumarate (19) supplemented with casamino acid (2 mg/ml), tryptophan (20 μg/ml), and ampicillin (50 μg/ml) at 37 °C for the production of wild-type and mutant PA c_{551} proteins.

Introduction of Mutations into PA c_{551} Gene—Two methods for site-directed mutagenesis were used to introduce a series of mutations in PA c_{551}. The first method was the PCR overlap extension technique (20, 21) that was used for the mutations of Phe-7 to Ala/Val-13 to Met (F7A/V13M), Phe-34 to Tyr/Gln-37 to Arg/Glu-43 to Tyr (F34Y/Q37R/E43Y), and Val-78 to Ile (V78I). The conditions for the PCR were as follows: incubation at 94 °C for 2 min followed by 28 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, with a final step of 5 min at 72 °C. Mutations of Phe-7 to Ala (F7A), Val-13 to Met (V13M), Phe-34 to Tyr (F34Y), Gln-37 to Arg (Q37R), Glu-43 to Tyr (E43Y), F34Y/Q37R, F34Y/E43Y, and Q37R/E43Y were introduced by the PCR-based kit, Mutan-Super Express Km (Takara Shuzo). All the PCR products were purified and digested with *Eco*RI and *Pst*I, and then ligated into the corresponding restriction sites of pKK223–3. The DNA sequences of the entire PCR products were confirmed by the dideoxy-chain termination method using ABI Prism model 310 DNA sequencer.

Production of PA c_{551} Proteins—The transformed *E. coli* cells harboring the wild-type or mutant PA c_{551} gene were harvested from the anaerobic culture. Periplasmic protein fractions were recovered by cold osmotic shock, and membrane and cytoplasmic fractions were obtained as described previously (11). Cytochromes *c* were specifically detected on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels by a heme staining procedure (22). The expressed PA c_{551} proteins in the periplasmic fraction were purified by Hi Trap Q or Mono Q column chromatography (Amersham Pharmacia Biotech), both eluted by 10 mM Tris-HCl buffer (pH 8.0) with an NaCl concentration gradient (0–100 mM), followed by a Superdex 75 column equilibrated and eluted with 50 mM ammonium acetate buffer (pH 7.0). Protein concentrations were determined by the Bio-Rad protein assay kit with bovine serum albumin as a standard. The N-terminal amino acid sequence of the purified wild-type PA c_{551} expressed in the *E. coli* periplasm was determined by automatic sequencer (Applied Biosystems model 470A).

UV-visible and Circular Dichroic (CD) Spectra—The UV-visible and CD spectra were measured on Hitachi U-3300 and Jasco J-720 machines, respectively. The protein samples were dissolved in water (pH 5.0 adjusted with HCl), the same conditions as used for thermal denaturation experiments.

NMR Measurements—Uniformly ^{15}N-labeled PA c_{551} was obtained from an anaerobic culture with $(^{15}NH_4)_2SO_4$ (99.3%, Shoko Co., Ltd.) and ^{15}N-labeled Algal amino acid mixture (98.2%, Shoko Co., Ltd.) used as nitrogen sources. The protein sample (~1 mM) was dissolved in 90% H_2O, 10% D_2O (pH 5.0 adjusted with HCl), and reduced with sodium dithionite. Three types of NMR measurements were carried out at 25 °C with a Varian Unity Inova 600 spectrometer: two-dimensional ^{1}H-^{15}N HSQC (23), three-dimensional ^{15}N-edited nuclear Overhauser effect spectroscopy-HSQC (24) with a mixing time of 100 ms, and three-dimensional ^{15}N-edited total correlation spectroscopy-HSQC (24) spectra with a mixing time of 40 ms.

Protein Thermostability—The wild-type and mutant PA c_{551} proteins (10 μg/ml protein concentration in water, pH 5.0 adjusted with HCl) were subjected to the thermal melting profile analysis by monitoring the changes of CD spectra at 222 nm as described previously (15) with slight modifications. The temperature of the protein solution was continuously raised from 25 °C to 100 °C at the rate of 50 °C per 60 min in the absence of guanidine hydrochloride (GdnHCl) and from 15 °C to 90 °C at the same rate in the presence of 1.5 M GdnHCl. The temperature in the cell was controlled using a Jasco PT343 thermoelectric temperature controller.

Denaturation with GdnHCl—The stability against GdnHCl denaturation of wild-type PA c_{551} or three mutants with F7A/V13M, F34Y/E43Y, or V78I substitutions was assayed. The proteins (10 μg/ml) were incubated in the diluted HCl water (pH 5.0) with varying concentrations of GdnHCl at 25 °C for 2 h before the measurement in order to equilibrate the proteins with the denaturant. The CD ellipticity at 222 nm of the protein solutions was measured at 25 °C.

[2] J. Cole, personal communication.

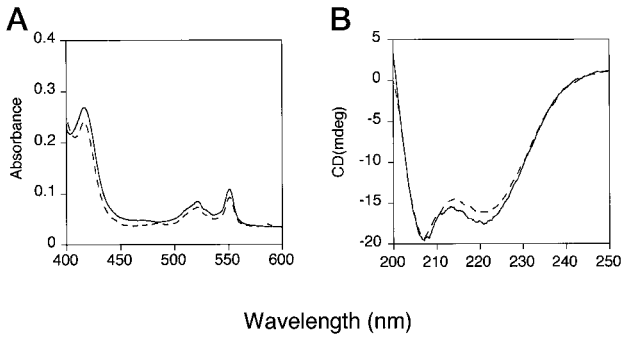

FIG. 1. **UV-visible and CD spectra.** UV-visible (*A*) and CD (*B*) spectra of wild-type PA c_{551} from *E. coli* (——) and *P. aeruginosa* (- - -) at 25 °C, pH 5.0 are shown.

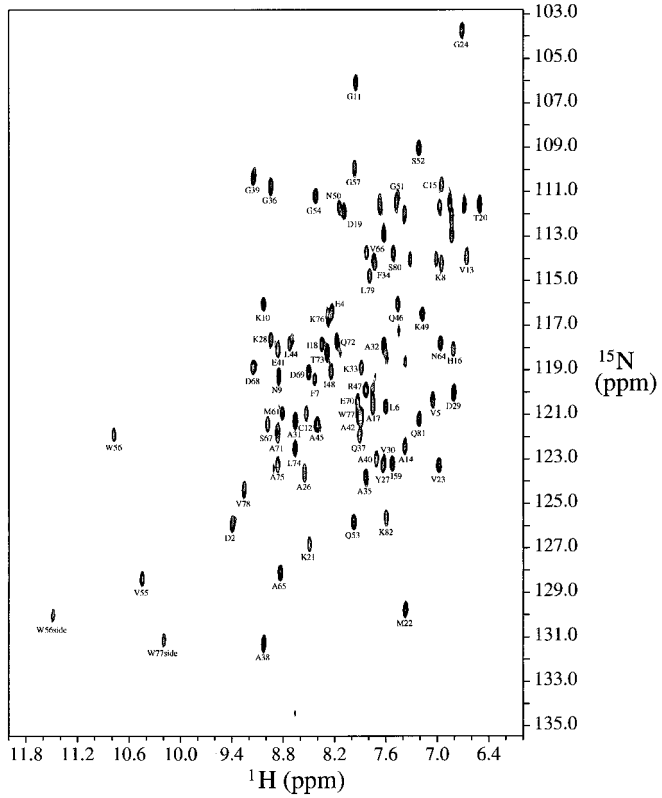

FIG. 2. **^{1}H-^{15}N HSQC spectrum of the reduced form of the PA c_{551} from *E. coli*.** The NMR data were processed using the software nmrPipe and nmrDraw (38), and the analysis was assisted by the software Pipp (39). Backbone signal assignment on ^{1}H-^{15}N HSQC spectrum was achieved based on the sequence-specific NOE connectivity (40). The cross-peaks are labeled according to the sequential assignment.

Materials—Restriction enzymes, *Taq* polymerase, T4 DNA ligase, and other reagents for DNA handling were purchased from Takara Shuzo or Toyobo. The authentic PA c_{551} protein from *P. aeruginosa* and GdnHCl were purchased from Sigma. All other chemicals used were of the highest grade commercially available.

RESULTS

Expression of PA c_{551} in the Periplasm of E. coli—The wild-type PA c_{551} protein expressed in *E. coli* JCB7120 strain was fully recovered in the cold osmotic shock fluid containing the periplasmic protein fraction, but not in the membrane and cytoplasmic fractions. The expressed protein had covalently attached heme as judged by heme staining following separation by SDS-polyacrylamide gel electrophoresis (data not shown). The production yield of holo-PA c_{551} in *E. coli* reached 8 mg/1 liter of culture (equal to 30% of the total periplasmic protein)

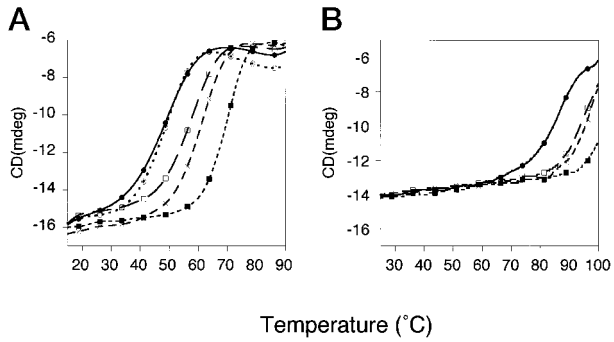

FIG. 3. **Melting profiles of wild-type and mutant PA c_{551} proteins.** Profiles for the wild-type PA c_{551} from *E. coli* (●), and *P. aeruginosa* (○), the mutants F7A/V13M (×), F34Y/E43Y(■), and V78I (□) are shown in the presence (*A*) and in the absence (*B*) of 1.5 M GdnHCl.

calculated by using a millimolar coefficient for the α band at 551 nm (21 mM^{-1} cm^{-1}). All the mutant proteins tested in this study could be obtained in the *E. coli* periplasm at almost the same yield as the wild-type.

The N-terminal amino acid sequence of the wild-type PA c_{551} protein expressed in the *E. coli* periplasm was determined as Glu-Asp-Pro-Glu-Val-Leu-Phe-Lys-Asn-Lys-Gly, which was identical to that of the authentic protein purified from the native organism.

Spectroscopy—The UV-visible (400–600 nm) spectrum of dithionite-reduced wild-type PA c_{551} protein expressed in *E. coli* showed absorption maxima at 417, 521, and 551 nm, which are characteristic features of authentic PA c_{551} protein (Fig. 1*A*). The far-ultraviolet CD (200–250 nm) spectrum of the air-oxidized form of the wild-type was also the same as that of the authentic protein, having an absorption peak at 222 nm (Fig. 1*B*). The same properties in UV-visible and CD spectra were obtained from all the mutant PA c_{551} proteins used in this study (data not shown). Furthermore, the ^{1}H-^{15}N HSQC spectrum of the dithionite-reduced wild-type protein was essentially the same as that of authentic protein (Fig. 2) (25). These results together suggested that the wild-type PA c_{551} expressed in *E. coli* folded correctly and the mutant proteins did not markedly differ in terms of the three-dimensional structure.

Assay Condition for Thermostability—The wild-type PA c_{551} from the native organism and *E. coli* both had the same cooperative melting transition with a T_m value of 50.4 °C in the presence of 1.5 M GdnHCl at pH 5.0 (Fig. 3*A*). The T_m values of all the mutants could be determined in the presence of the same concentration of the denaturant (Fig. 3*A* and Table I). Therefore, we carried out the thermal denaturation assays under these conditions throughout the present study. By contrast, in the absence of GdnHCl, the T_m values of the mutant proteins could not be determined because they did not reach a completely denatured state even at 100 °C (Fig. 3*B*).

Substitutions of Phe-7 and Val-13—In PA c_{551}, a small cavity exists around the side chains of Phe-7 and Val-13, which correspond to Ala and Met, respectively, in HT c_{552}. The three-dimensional structure of HT c_{552} shows that the side chains of the Ala and Met fill this cavity (14). The double mutation F7A/V13M in PA c_{551} caused increased thermostability compared with the wild-type (ΔT_m: 12.0 °C; Fig. 3*A* and Table I). Each single mutation, F7A and V13M, enhanced the thermostability essentially in an additive manner, the individual ΔT_m values being 9.5 and 3.2 °C, respectively (Fig. 3*A* and Table I, see ΔT_m and $\Delta T_m{}^{hyp}$ values of F7A/V13M mutant).

Substitutions of Phe-34, Gln-37, and Glu-43—Phe-34 and Glu-43 in PA c_{551} are both substituted by Tyr residues in HT c_{552}. The two Tyr aromatic side chains are closely located in the three-dimensional structure of HT c_{552} and suggested to have

TABLE I
Parameters characterizing the thermal denaturation of the mutant PA c_{551}

The temperature of the midpoint of the transition (T_m) and the enthalpy change during unfolding at T_m (ΔH) were calculated from curve fitting of the resulting CD values *versus* the temperature data on the basis of van't Hoff analysis. This curve fitting was achieved using the function of a least-square analysis in the software MATHEMATICA (Wolflam Inc.). The entropy change during unfolding at T_m (ΔS) was calculated using the equation, $\Delta S = \Delta H/T_m$. The differences in the free energy changes of unfoldings between the mutant proteins and wild-type at the wild-type T_m ($\Delta\Delta G_m$) were calculated using the equation given by Becktel and Schellman (41), $\Delta\Delta G_m = \Delta T_m{}^* \Delta S$ (wild-type), where ΔT_m is the difference in T_m values between the mutant and wild-type proteins, and ΔS_m (wild-type) is the entropy change of the wild-type protein at the T_m. The hypothetical ΔT_m value ($\Delta T_m{}^{hyp}$) was calculated for each mutant protein with multiple mutations assuming that the effect of each amino acid replacement on the protein stability is independent and cumulative.

Protein	ΔH	ΔS	T_m	ΔT_m	$\Delta T_m{}^{hyp}$	$\Delta\Delta G_m$
	kcal/mol	*kcal/mol/K*	*°C*	*°C*	*°C*	*kcal/mol*
Wild-type	37.6	0.116	50.4			
F7A	48.2	0.145	59.9	9.5		1.1
V13M	43.8	0.134	53.6	3.2		0.4
F7A/V13M	46.7	0.139	62.4	12.0	12.7	1.4
F34Y	61.2	0.180	66.4	16.0		1.9
Q37R	51.3	0.156	54.7	4.3		0.5
E43Y	57.4	0.174	55.5	5.1		0.6
F34Y/Q37R	54.5	0.162	62.9	12.5	20.3	1.5
F34Y/E43Y	63.7	0.185	70.7	20.3	21.1	2.4
Q37R/E43Y	46.2	0.141	54.7	4.3	9.4	0.5
F34Y/Q37R/E43Y	56.4	0.165	57.9	17.5	25.4	2.0
V78I	43.8	0.132	58.8	8.4		1.0

hydrophobic interaction with one another (14). The T_m values of PA c_{551} with single F34Y and E43Y mutations were increased by 16.0 and 5.1 °C, respectively, compared with the wild-type (Fig. 3*A* and Table I). The simultaneous mutation (F34Y/E43Y) caused enhanced thermostability, which was contributed by each single mutation in a cumulative manner (ΔT_m: 20.3 °C, $\Delta T_m{}^{hyp}$: 21.1 °C, Fig. 3*A* and Table I).

Although the single Q37R mutation in PA c_{551} reproducibly made a small contribution to the increased stability (ΔT_m value was 4.3 °C, Table I), the T_m values of PA c_{551} protein with F34Y/Q37R, Q37R/E43Y, and F34Y/Q37R/E43Y mutations (ΔT_m: 12.5, 4.3, and 17.5 °C, respectively; Table I) were each significantly lower than those with F34Y, E43Y, and F34Y/E43Y, respectively.

Substitution of Val-78—The region around Val-78 in PA c_{551}, interacting with heme hydrophobically, should become more hydrophobic if Val were substituted by Ile as in HT c_{552}; this is because Ile has one additional methyl group in the side chain compared with that of Val. As expected, the V78I mutation in PA c_{551} caused an 8.4 °C elevation of the T_m value compared with that of the wild-type (Fig. 3A and Table I).

Stability against GdnHCl Denaturation—We also tested whether F7A/V13M, F34Y/E43Y, and V78I mutations in PA c_{551} stabilize the structure against GdnHCl denaturation. Fig. 4 shows GdnHCl-induced denaturation curves and plots of ΔG *versus* GdnHCl concentration around the midpoint of denaturation (C_m). The denaturation curve of the wild-type showed that 1.5 M GdnHCl did not denature the protein structure. Thus, in the thermal denaturation experiments, the temperature of the protein samples can be ramped from the non-denatured condition in the presence of 1.5 M GdnHCl. The C_m values of the mutant proteins (F7A/V13M, F34Y/E43Y, and V78I) were elevated as compared with that of the wild-type (ΔC_m: 0.52, 0.73, and 0.19 M, respectively; Table II). Among these mutants, F34Y/E43Y was the most stable, followed by the F7A/V13M, and then the V78I protein, as judged by the comparison of ΔC_m and $\Delta\Delta G^{H_2O}$ values. This order of the stability

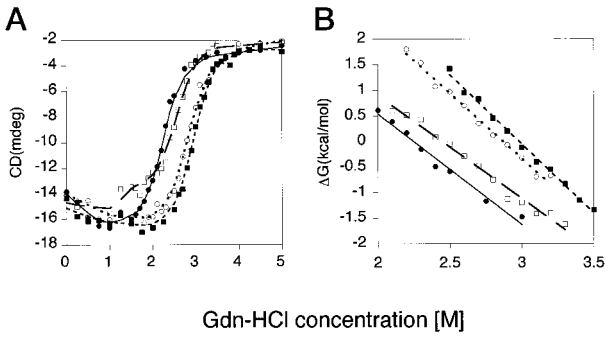

FIG. 4. **GdnHCl-induced denaturation of wild-type and mutant PA c_{551} proteins.** *A*, denaturation curves are shown as a function of GdnHCl concentration for the wild-type PA c_{551} (●), the mutants F7A/V13M (○), F34Y/E43Y (■), and V78I (□). *B*, the free energy changes of unfolding (ΔG) are shown as a function of the GdnHCl concentration around the midpoint of the transition. Symbols are the same as those in *panel A*.

TABLE II
Parameters characterizing GdnHCl denaturation

The difference in free energy change between the folded and unfolded states (ΔG) was calculated as described by Pace (42). The free energy change in H_2O (ΔG^{H_2O}) and the dependence of ΔG on the GdnHCl concentration (m) were determined by a least-squares fit of the data from the transition region using the equation: $\Delta G = \Delta G^{H_2O} - m[GdnHCl]$ (42). The midpoint of the GdnHCl denaturation (C_m) was the concentration of GdnHCl at which the ΔG value became 0. The differences in C_m (ΔC_m) and ΔG^{H_2O} ($\Delta\Delta G^{H_2O}$) between the wild-type and mutant proteins were calculated by subtracting the values of the wild-type from those of mutants.

Protein	C_m	ΔC_m	m	ΔG^{H_2O}	$\Delta\Delta G^{H_2O}$
	M	*M*	*kcal · mol/M*	*kcal/mol*	*kcal/mol*
Wild-type	2.25		2.17	4.88	
F7A/V13M	2.87	0.52	2.56	7.34	2.46
V78I	2.44	0.19	2.02	4.94	0.06
F34Y/E43Y	2.98	0.73	2.71	8.09	3.21

was same as that from the heat denaturations of these mutants.

DISCUSSION

In this study we developed an expression system for PA c_{551} using *E. coli* JCB7120 strain as a host organism. The correctly processed wild-type PA c_{551} expressed in the periplasm of *E. coli* has the same spectral properties in CD, UV-visible, and NMR, and also has the same thermostability as the authentic protein, indicating that the protein is in "native" state. In previous studies, the PA c_{551} gene on extra-chromosomal plasmids could be expressed heterologously in *Pseudomonas putida* (26) and in the original organism (18). The holo-PA c_{551} formation was also observed in the periplasm of other *E. coli* strains.[3] The expression level in the present *E. coli* strain was the highest relative to any previous studies that demonstrated heterologous expression of cytochromes *c* in other *E. coli* strains (16, 27, 28),[4] The efficient production level and easy purification procedure from the *E. coli* periplasm enabled us to obtain ^{15}N-labeled PA c_{551} for heteronuclear NMR spectroscopy (detailed structural analysis is in progress). Furthermore, this system will facilitate other studies requiring large amounts of the protein sample such as differential scanning calorimetry and x-ray crystallography. The JCB7120 strain was chosen because it expresses higher levels of endogenous *c*-type cytochromes than other strains (29).[2] The basis for this is not known, but it is seemingly not a consequence of enhanced expression of *c*-type cytochrome biogenesis genes (30) because

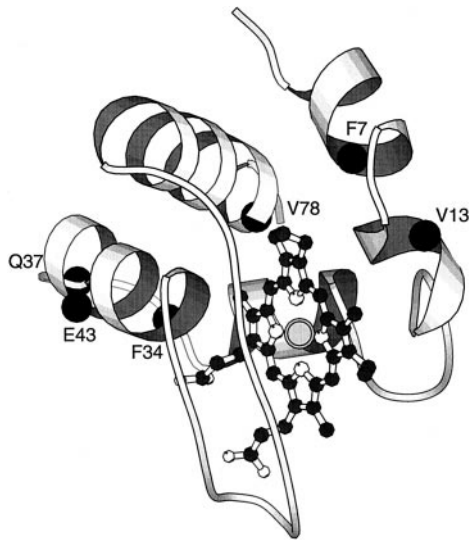

FIG. 5. **Schematic view of mutation points in PA c_{551} protein.** The positions of α carbon atoms of the mutated residues are shown by *closed circles*. Heme iron is indicated as *double lined circle*. The atomic coordinates for PA c_{551} were taken from Protein Data Bank (identification code 451C) (43). The figure was prepared by using the program Molscript (44).

the JCB7120 strain also produces high level of cytoplasmically expressed HT c_{552} (31), a process that is independent of the biogenesis genes.

Although 35 amino acid residues are substituted between HT c_{552} and PA c_{551}, we have postulated, from structure comparison between the proteins, that a few amino acid residues in the three regions formed by Phe-7/Val-13, Phe-34/Gln-37/Glu-43, and Val-78 (Fig. 5; see Fig. 7 in Ref. 14 for the detailed structures) are important for stability. Therefore, we systematically introduced the mutations in these regions of PA c_{551} modeled by the corresponding residues in HT c_{552}. All the mutations tested in this study resulted in the increased stability, although HT c_{552} was still more stable than these mutants; its T_m and C_m values were 91.8 °C and 4.49 M, respectively, assayed under the same condition as used for the PA c_{551} proteins.[5] It is notable that the present findings with a mainly α helical protein contrast with those made with the β-sheet rubredoxins from *Pyrococcus furiosus* (thermophile) and *Clostridium pasteurianum* (mesophile) (32). For these iron-sulfur proteins substantial exchanges of linear sequence, rather than individual mutations, have been shown to be required to enhance thermostability, implying that many small interactions cumulatively contribute to large increases in the stability.

The increased stability of the PA c_{551} with F7A, V13M, F7A/V13M, or V78I mutations indicates that the void spaces in PA c_{551} formed by the side chains of the original residues destabilize the protein structure. Therefore, the mutations designed to fill the void space were effective in increasing stability. It has been suggested in other proteins that higher stability can be achieved when Val is substituted by Ile, having one additional methyl group (33, 34) as found in the PA c_{551} V78I mutant. The stabilization by the E43Y mutation in PA c_{551} could also be attributed to tighter hydrophobic packing between the introduced Tyr residue and Phe-34, Ala-40, or Leu-44, among which the latter two residues are conserved in HT c_{552}.

The $\Delta\Delta G_m$ value for the F34Y mutant is 1.9 kcal/mol, which is comparable to the free energy of the hydrogen bond (2~4 kcal/mol). Consistent with this calculation, three-dimensional

[3] Y. Sambongi and S. J. Ferguson, unpublished results.
[4] Y. Sambongi, unpublished results.
[5] J. Hasegawa, unpublished results.

molecular modeling of PA c_{551} with the F34Y mutation suggest that the η oxygen atom of the introduced Tyr-34 forms a hydrogen bond with the guanidyl base of Arg-47 in PA c_{551}: the distance between these atoms is estimated to be 3.2 Å. However, Lys-45 in HT c_{552} (corresponding to the PA c_{551} Arg-47) does not form a hydrogen bond with the "original" Tyr residue. If Lys-45 could be substituted by Arg in HT c_{552}, much higher thermostability should be obtained in the thermophilic protein. The ΔT_m value for the PA c_{551} F34Y mutant (16 °C) is the largest among those of the single amino acid mutants of PA c_{551}, and this thermostabilization is one of the most dramatic observed for a single substitution in any protein. It is equivalent to that of a yeast iso-1-cytochrome *c* mutant, which exhibits the highest elevation of T_m ever observed (35).

The NMR solution structure of HT c_{552} indicates that Arg-35 and the two Tyr residues (corresponding to Gln-37, Phe-34, and Glu-43 in PA c_{551}, respectively) form aromatic-amino interactions (14). These interactions have been suggested to cause the higher thermostability of HT c_{552}. However, the Q37R mutation negatively affected thermostability of the three PA c_{551} proteins with F34Y, E43Y, or F34Y/E43Y mutations. This observation clearly indicates that the aromatic-amino interaction(s) are formed between the introduced Arg and Tyr residues as in HT c_{552}, but these interactions may disturb the hydrogen bond formation between Tyr-34 and Arg-47, and/or the hydrophobic interactions between Tyr-43 and Phe-34, Ala-40, Tyr-41, or Leu-44.

In this study, successful enhancement of protein stability has been achieved by filling small void spaces, increasing hydrophobicity, and generation of a hydrogen bond in the three local regions of PA c_{551}. *Hydrogenophilus thermoluteolus* (formerly *Pseudomonas hydrogenothermophila*; Ref. 36), which grows optimally at 52 °C, has a homologous cytochrome c_{552} (HP c_{552}, having 65% amino acid identity to HT c_{552}), although the partial sequence (60 amino acids) is only available. HP c_{552} has been shown to have high thermostability like HT c_{552} (37). It should be noted that, in the HP c_{552} protein, corresponding amino acid residues to PA c_{551} Phe-7, Val-13, and Phe-34 are, respectively, Ala, Met, and Tyr as found in HT c_{552}. These findings may support the proposition that the substitutions of Phe to Ala, Val to Met, and Phe to Tyr at these positions play important roles in the protein stability, assuming that HP c_{552} protein has a three-dimensional structure similar to that of HT c_{552} and PA c_{551}. Our study strongly indicates that the HT c_{552} can be used as an ideal model protein, it is 26 residues smaller than the yeast iso-1-cytochrome *c*, for elucidating the roles of amino acid residues in protein stability by mutating the mesophilic homologue, PA c_{551} protein.

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Stabilization of *Pseudomonas aeruginosa* Cytochrome c_{551} by Systematic Amino Acid Substitutions Based on the Structure of Thermophilic *Hydrogenobacter thermophilus* Cytochrome c_{552}

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