Stabilization of Pseudomonas aeruginosa Cytochrome c₅₅₁ by Systematic Amino Acid Substitutions Based on the Structure of Thermophilic Hydrogenobacter thermophilus Cytochrome c₅₅₂*

(Received for publication, July 16, 1999, and in revised form, October 8, 1999)

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A heterologous overexpression system for mesophilic Pseudomonas aeruginosa holocytochrome c₅₅₁ (PA c₅₅₁) was established using Escherichia coli as a host organism. Amino acid residues were systematically substituted in three regions of PA c₅₅₁ with the corresponding residues from thermophilic Hydrogenobacter thermophilus cytochrome c₅₅₂ (HT c₅₅₂), which has similar main chain folding to PA c₅₅₁, but is more stable to heat. Thermodynamic properties of PA c₅₅₁ 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 c₅₅₁ with HT c₅₅₂. 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 c₅₅₂. 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 c₅₅₁ can be achieved through a few amino acid substitutions determined by molecular modeling with reference to the structure of HT c₅₅₂. The higher stability of HT c₅₅₂ 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 threedimensional 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 c₅₅₂ (HT c₅₅₂) from a thermophilic hydrogen oxidizing bacterium, Hydrogenobacter thermophilus that grows optimally at 70 °C, is an 80-amino acid protein with a heme. HT c₅₅₂ has 56% sequence identity to an 82-amino acid monoheme cytochrome c₅₅₁ (PA c₅₅₁) 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 c₅₅₂ is more stable to heat than PA c₅₅₁ (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 c₅₅₂ compared with PA c₅₅₁. PA c₅₅₁ and HT c₅₅₂ are thus very suitable proteins for identifying substitutions of amino acid residues that endow stability.

Here we report that holo-PA c₅₅₁, 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 c₅₅₁ could be significantly increased through selected mutations, which had been chosen by molecular modeling with reference to corresponding amino acid residues in HT c₅₅₂. We discuss the structural origins of higher stability of HT c₅₅₂.

EXPERIMENTAL PROCEDURES

Bacterial Strain, Plasmids, and Growth Condition—The EcoRI-PstI gene fragment CP1, encoding the 22-amino acid signal sequence and

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* This work was supported in part by grants from the Japanese Ministry of Education, Science and Culture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ Supported by a fellowship from the Japan Society for the Promotion of Science.

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1 The abbreviations used are: HT c₅₅₂, ferrocytochrome c₅₅₂ from H. thermophilus; GdnHCl, guanidine hydrochloride; PCR, polymerase chain reaction; PA c₅₅₁, ferrocytochrome c₅₅₁ from P. aeruginosa; HP c₅₅₂, ferrocytochrome c₅₅₂ 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 furamate (19) supplemented with casamino acid (2 mg/ml), tryptophan (20 mg/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/Glu-37 to Arg (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), Gly-37 to Arg (G37R), Glu-43 to Tyr (E43Y), F34Y/Q37R, F34Y/E43Y, and Q37R/E43Y were introduced by the PCR-based kit, MutanSuper Express Km (Takara Shuzo). All the PCR products were purified and digested with EcoRI and PstI, 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 TrapolQ 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-3000 and Jasco J-720 machines respectively. The protein samples were redissolved in water (pH 5.0 adjusted with HCl), the same conditions as used for thermal denaturation experiments.

NMR Measurements—Uniformly ^15N-labeled PA[c][551] was obtained from an anaerobic culture with ((^15)NH4)2SO4 (99.3%, Shoko Co., Ltd.) and ^13C-labeled Algal amino acid mixture (98.2%, Shoko Co., Ltd.) used as nitrogen sources. The protein sample (~1 mg) was dissolved in 90% H2O, 10% D2O (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 ^15N-HSQC (23), three-dimensional ^13C-edited nuclear Overhauser effect spectroscopy-HSQC (24) with a mixing time of 100 ms, and three-dimensional ^15N-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) in 5% GdnHCl 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.

*J. Cole, personal communication.

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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. 1H-^15N 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 Pip (39). Backbone signal assignment on 1H-^15N 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 further purified in the cold osmotic shock flux 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/liter of culture (equal to 30% of the total periplasmic protein)}
calculated by using a millimolar coefficient for the \( \alpha \) band at 551 nm (21 \text{ m}^{-1} \text{ cm}^{-1}). All the mutant proteins tested in this study could be obtained in the \( E. \text{coli} \) 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 \( E. \text{coli} \) 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 \( E. \text{coli} \) showed absorption maxima at 417, 521, and 551 nm, which are characteristic features of authentic PA \( c_{551} \) protein (Fig. 1A). 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. 1B). 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 \( ^1 \text{H},^15 \text{N} \) 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 \( E. \text{coli} \) 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 \( E. \text{coli} \) 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. 3A). The \( T_m \) values of all the mutants could be determined in the presence of the same concentration of the denaturant (Fig. 3A 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. 3B).

**Substitutions of Phe-7 and Val-19—**In PA \( c_{551} \), a small cavity exists around the side chains of Phe-7 and Val-19, 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 \( F7A/V19M \) in PA \( c_{551} \) caused increased thermostability compared with the wild-type \( \Delta T_m \): 12.0 °C (Fig. 3A and Table I). Each single mutation, \( F7A \) and \( V19M \), enhanced the thermostability essentially in an additive manner, the individual \( \Delta T_m \) values being 9.5 and 3.2 °C, respectively (Fig. 3A and Table I), see \( \Delta T_m \) and \( \Delta T_m \) values of \( F7A/V19M \) 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 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. 3A and Table I). The simultaneous mutation (F34Y/E43Y) caused enhanced thermostability, which was contributed by each single mutation in a cumulative manner (\( \Delta T_m \): 20.3 °C, \( \Delta T_m \) values: 21.1 °C, Fig. 3A and Table I).

Although the single Q37R mutation in PA \( c_{551} \) reproducibly made a small contribution to the increased stability \( \Delta T_m \) 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 \( \Delta T_m \) values: 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 \( \Delta G \) 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 experiment, the temperature of the samples can be raised from the 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 \((\Delta C_{m} ; 0.52, 0.73, \) and 0.19 M, respectively; Table III). 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 \( \Delta C_{m} \) and \( \Delta G^{H_{2}O} \) values. This order of the stability

**TABLE I**

| Protein | \( \Delta H \) | \( \Delta S \) | \( T_m \) | \( \Delta T_m \) | \( \Delta T_m \) | \( \Delta G_m \) |
|---------|--------------|------------|--------|-------------|-------------|-------------|
| 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 | 1.4 |
| F34Y | 61.2 | 0.180 | 68.4 | 16.0 | 1.9 |
| F34Y/E43Y | 63.7 | 0.185 | 70.7 | 20.3 | 2.4 |
| 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 | 1.5 |
| F34Y/E43Y | 46.2 | 0.141 | 54.7 | 4.3 | 0.5 |
| F34Y/Q37R/E43Y | 56.4 | 0.165 | 57.9 | 17.5 | 2.0 |
| V78I | 43.8 | 0.132 | 58.8 | 8.4 | 1.0 |

**Fig. 3. Melting profiles of wild-type and mutant PA \( c_{551} \) proteins.** Profiles for the wild-type PA \( c_{551} \), from \( E. \text{coli} \) (●), and \( P. \text{aeruginosa} \) (□), the mutants \( F7A/V13M \) (○), \( F34Y/E43Y \) (■), and \( V78I \) (□) are shown in the presence (A) and in the absence (B) of 1.5 M GdnHCl.
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 H2O (ΔG(H2O)) 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: ΔG = ΔG(H2O) + mΔG(H2O)/2 for the GdnHCl at which the ΔG value became 0. The differences in Cm (ΔCm) and ΔG(H2O) (ΔΔG(H2O)) between the wild-type and mutant proteins were calculated by subtracting the values of the wild-type from those of mutants.

### Table II

Parameters characterizing GdnHCl denaturation

| Protein          | Cm(mM) | m  | ΔG(H2O)(kcal/mol) | ΔΔG(H2O)(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 c551 using E. coli JCB7120 strain as a host organism. The correctly processed wild-type PA c551 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 NMR, and also has the same thermostability as the authentic PA c551, the midpoint of the GdnHCl denaturation (Cm) was 91.8 °C and 4.49 M, respectively, assayed under the same condition as used for the PA c551 proteins. 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 c551 with F7A, V13M, F7A/V13M, or V78I mutations indicates that the void spaces in PA c551 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 c551 V78I mutant. The stabilization by the E43Y mutation in PA c551 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 c552.

The ΔΔG(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 structures

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3. Y. Sambongi and S. J. Ferguson, unpublished results.
4. Y. Sambongi, unpublished results.
5. J. Hasegawa, unpublished results.
molecular modeling of PA c551 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 c551; the distance between these atoms is estimated to be 3.2 Å. However, Lys-45 in HT c552 (corresponding to the PA c551 Arg-47) does not form a hydrogen bond with the “original” Tyr residue. If Lys-45 could be substituted by Arg in HT c552, much higher thermostability should be obtained in the thermophilic protein. The ΔT_m value for the PA c551 F34Y mutant (16 °C) is the largest among those of the single amino acid mutants of PA c551, 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 c552 indicates that Arg-35 and the two Tyr residues (corresponding to Gln-37, Phe-34, and Lys-45) contribute to the high thermostability of HT c552, much higher than that of a yeast iso-1-cytochrome c552. Our study strongly indicates that the HT c552 can be used as an ideal model protein, it is 26 residues smaller than HT c551, having 65% amino acid identity to HT c551 Phe-7, Val-13, and Phe-34, which is also observed (35).

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 c551. Hydrogenophilus thermoluteolus (formerly Pseudomonas hydrogenothermophila; Ref. 36), which grows optimally at 52 °C, has a homologous cytochrome c with a higher thermostability of HT c552. The NMR solution structure of HT c552 (37), 65–69

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