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| **Relation**  |                                                                                               |
Selected Mutations in a Mesophilic Cytochrome \(c\) Confer the Stability of a Thermophilic Counterpart*

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Mesophilic cytochrome \(c_{551}\) of \(Pseudomonas\) \(aeruginosa\) (PA \(c_{551}\)) became as stable as its thermophilic counterpart, \(Hydrogenobacter\) \(thermophilus\) cytochrome \(c_{552}\) (HT \(c_{552}\)), through only five amino acid substitutions. The five residues, distributed in three spatially separated regions, were selected and mutated with reference to the corresponding residues in HT \(c_{552}\) through careful structure comparison. Thermodynamic analysis indicated that the stability of the quintuple mutant of PA \(c_{551}\) could be partly attained through an enthalpic factor. The solution structure of the mutant showed that, as in HT \(c_{552}\), there were tighter side chain packings in the mutated regions. Furthermore, the mutant had an increased total accessible surface area, resulting in great negative hydration free energy. Our results provide a novel example of protein stabilization in that limited amino acid substitutions can confer the overall stability of a natural highly thermophilic protein upon a mesophilic molecule.

Heat-stable proteins from thermophilic bacteria usually exhibit main chain foldings similar to those of mesophilic counterparts. Mutational studies on mesophilic proteins modeled with respect to thermophilic counterparts have proved that specific side chain interactions in the thermophiles are partially responsible for the higher stability (1–3). Thermodynamic analysis has also indicated that a thermophilic protein can be stabilized through global interaction throughout the molecule (4). It remains enigmatic as to how many amino acid substitutions contribute to the stability of a natural thermophilic protein (5). In some cases, a mesophilic protein only acquires the stability of the thermophilic counterpart after substantial exchanges of a linear sequence; groups of individual mutations are not sufficient (6). Multiple mutations in mesophilic proteins that completely increase the stability to the levels of thermophilic counterparts would provide important information about relationships between local side chain interactions and overall protein stability and demonstrate that the thermophilic character can depend on a limited number of strong noncovalent interactions.

Cytochrome \(c\) is a powerful tool for characterizing protein stability because structural information on a variety of cytochromes \(c\) is available, and heterologous expression systems for holoproteins have been established (7). Cytochrome \(c_{551}\) (PA \(c_{551}\)) from a mesophile, \(Pseudomonas\) \(aeruginosa\), and cytochrome \(c_{552}\) (HT \(c_{552}\)) from a thermophile, \(Hydrogenobacter\) \(thermophilus\), are 82- and 80-amino acid proteins, respectively, each with a covalently attached heme. These proteins exhibit 56% sequence identity (8) and almost the same main chain structures (9), but HT \(c_{552}\) exhibits much higher stability compared with PA \(c_{551}\) (10). On a structural comparison between HT \(c_{552}\) (9) and PA \(c_{551}\) (11), we identified three distal regions responsible for the higher stability of the former (9). The single mutation Val-78 to Ile (V78I), and two double mutations Phe-7 to Ala/Val-13 to Met (F7A/V13M) and Phe-34 to Tyr/Glu-43 to Tyr (F34Y/E43Y), chosen with reference to HT \(c_{552}\), in these three regions of PA \(c_{551}\) have each been shown to increase protein stability (1).

In the present study, the five mutations were introduced together into PA \(c_{551}\). Thermodynamic analysis showed that the quintuple mutant of PA \(c_{551}\) was as stable as HT \(c_{552}\). In order to provide a molecular basis for understanding protein stabilization, we have determined the solution structure of the quintuple mutant. We discuss factors contributing to protein stability in conjunction with structural analyses of HT \(c_{552}\) and the wild-type and quintuple mutant PA \(c_{551}\) proteins.

**EXPERIMENTAL PROCEDURES**

**Protein Preparations**—Mutations were introduced into the PA \(c_{551}\) gene with a polymerase chain reaction-based kit, Mutan-Super Express Km (Takara, Kyoto, Japan), as described previously (1). Transformed \(Escherichia\) \(coli\) JCB7120 cells harboring PA \(c_{551}\) genes were harvested from an anaerobic culture, and the proteins used in this study were purified as described previously (1, 8). The concentrations of the purified protein solutions were determined spectrophotometrically using extinction coefficients of \(c_{255} = 25,200\) cm\(^{-1}\) M\(^{-1}\) and \(c_{415} = 20,400\) cm\(^{-1}\) M\(^{-1}\) for PA \(c_{551}\) and HT \(c_{552}\), respectively. The uniformly \(^{15}\)N- or \(^{13}\)C/\(^{15}\)N-labeled quintuple mutant PA \(c_{551}\) protein was obtained from an anaerobic culture with \(^{15}\)NH\(_2\) \(\cdot\) \(^{15}\)NO\(_3\) (99.3%), a \(^{15}\)N-labeled Algal amino acid.

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The atomic coordinates and structure factors (code 1DVV) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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1 The abbreviations used are: PA \(c_{551}\), ferrocyanochrome \(c_{551}\) from \(P.\) \(aeruginosa\); HT \(c_{552}\), ferrocyanochrome \(c_{552}\) from \(H.\) \(thermophilus\); GdnHCl, guanidine hydrochloride; HSCQC, heteronuclear single quantum correlation; NOESY, nuclear Overhauser effect spectroscopy; DQF-COSY, double quantum-filtered correlation spectroscopy; DSC, differential scanning calorimetry; ASA, accessible surface area; r.m.s., root mean square.

37824 This paper is available on line at http://www.jbc.org
acid mixture (98.2%), and a glycerol-13C3 and 15N-labeled Algal amino acid mixture (97.5%) as nitrogen and carbon sources. The labeled compounds were obtained from Shoko Co., Ltd. (Tokyo, Japan).

Guanosine Hydrochloride (GdnHCl) Denaturation—Proteins (10 µg/ml) were incubated in diluted HCl water (pH 5.0) with various concentrations of GdnHCl at 25 °C for 2 h before measurements in order to equilibrate the proteins with the denaturant. The CD ellipticity at 222 nm was monitored using a 1-cm path length cuvette with a heating rate of 1 K/min. Thermodynamic parameters, \( \Delta C_P \), and \( \Delta G \) were estimated from three independent measurements.

Thermal Denaturation—The temperature dependence of the CD ellipticity at 222 nm was measured using a 1-cm path length cuvette with a JASCO J-720 spectrophotometer with a PTS43 thermoelectric temperature controller. The data were fitted by nonlinear least-squares analysis with KaleidaGraph 3.0 (Synergy Software) employing the Marquard-Levenberg algorithm using a linear extrapolation model as described previously (12). \( C_m \) was the concentration of GdnHCl at which the free energy change value, \( \Delta G \), became 0.

Structure Calculation—Approximate interproton distances were obtained from simultaneous 1H-15N- and 15N-13C(H)-edited NOESY-HSQC (27) spectra. All NOE signal assignments were made through HCCCH-total correlation spectroscopy experiments (28). Stereo-specific assignments of the α-methyl protons of valine and β-methylene protons were made by analyzing HNHB (23), 15N-edited NOESY-HSQC (24), and DQF-COSY (25) spectra. All proton signals from the heme moiety were assigned according to the procedure of Keller and Wuthrich (26). The signals of carbons attached to heme protons were assigned with constant time13C-1H HSQC spectra employed the Marquard-Levenberg algorithm.

NMR Measurement—A protein sample (~1 mg) of the quintuple mutant was dissolved in a 90% H2O, 10% D2O or 99.99% D2O (v/v) solution (pH 5.0 adjusted with HCl), and then reduced with sodium dithionite. All NMR experiments were performed at 25 °C with a Varian UNITYInova 600 spectrometer. Sequential assignments of the backbone resonances of the polypeptide chain were achieved by means of sets of experiments, HNCA (17), CBCA(CO)NH (18), HNCO (19), HBCBCAC(CA)HA (20), and INHA (21). Protein side chain assignments were made through HCCH-total correlation spectroscopy experiments (22). Stereospecific assignments of the γ-methyl protons of valines and β-methylene protons were made by analyzing HNHB (23), 15N-edited NOESY-HSQC (24), and DQF-COSY (25) spectra. All proton signals from the heme moiety were assigned according to the procedure of Keller and Wuthrich (26). The signals of carbons attached to heme protons were assigned with a constant time13C-H HSQC spectrum (27). All data were processed using the software NMRPipe (28), and the data analysis was assisted by the software PIPP (29). The \( ^{1}H, ^{13}C \), and \( ^{15}N \) resonance assignments of the quintuple mutant have been deposited in the BioMagResBank, under accession number 4578.
were used for each hydrogen bond. Structures were calculated using the
YASASAP protocol (32) within X-PLOR 3.1 (33). The coordinates of the
quintuple mutant of PA c551 have been deposited in the Protein Data
Bank, under accession number 1DVV.

Calculation of the Accessible Surface Area (ASA) and Gibbs Free
Energy of Hydration for the Native State (GhN)—

Energy (kcal/mol)

Table II

Statistics of the 20 structures of the quintuple mutant protein
(SA) represents the 20 individual structures calculated with the
X-PLOR program. (SA)r is the refined structure obtained by energy
minimization of the mean structure obtained by simple averaging of the
coordinates of the SA structures. FNOE and Fvdw were calculated using
force constants of 50 kcal/mol/Å² and 200 kcal/mol/rad², respectively. Fvdw was calculated using a final value of 4 kcal/mol/Å² with the van der
Waals hard sphere radii set to 0.75 times those in the parameter set
PARALLHSA supplied with the X-PLOR program.

 Results and Discussion

Stability against GdnHCl Denaturation—The far-ultraviolet
CD spectrum of the quintuple mutant of PA c551 (F7A/V13M/
F34Y/E43Y/V78I) was nearly identical to that of the wild type
denatured state (Fig. 1A and Table I) and essentially the same as that of HT
552, respectively. Surfaces were classified into polar and nonpolar
components by regarding carbon and sulfur atoms as nonpolar and
oxygen and nitrogen as polar (35). GhN was calculated according to the
method proposed by Oobatake and Ooi (36).

Fig. 3. Structures of the quintuple mutant and wild-type PA
c551 proteins and HT c552. A, stereoview of the 20 structures of the
quintuple mutant, B, schematic representation of main chain folding of
the quintuple mutant (purple) overlaid with those of the wild-type PA
c551 (green) and HT c552 (red).

Fig. 2. DSC analysis of PA c551 and HT c552. A, molar heat capacity
curves in the presence of 1.5 M GdnHCl observed by DSC for the wild-type
PA c552 (○), the quintuple mutant PA c551 (○), and HT c552 (■). The solid lines represent the results of nonlinear least-squares best fits based on
the two-state model. B, heat capacity curves for wild-type PA c551 at pH 3.6 (○), 3.8 (○), and 4.0 (■). C, enthalpy change of denaturation measured
at different pH values versus the corresponding denaturation temperature.

from DSC measurements (see below and Table I), indicating
that thermal denaturation of these proteins proceeded in a
two-state manner.

Fig. 2A shows the heat capacity curves of the three proteins
measured by DSC. From these curves, thermodynamic parameters
were obtained as a function of temperature. We further
obtained the ΔCp value of the wild-type PA c551 from Tm-dependent
ΔH(Tm) measurements at pH 3.6, 3.8, and 4.0 in the
absence of GdnHCl (Fig. 2, B and C) (37). The ΔCp value
obtained from the \(T_m\)-dependent \(\Delta H\) value was 781 cal/mol/K, which was close to that obtained on nonlinear fitting of the CP curve at pH 5.0 in the presence of 1.5M GdnHCl (720 cal/mol/K; Table I). These results indicate that the \(\Delta H\) values obtained in this study are reliable ones. It has also been reported that the \(\Delta H\) values do not dramatically change in the presence of GdnHCl up to 2.0 M (38).

The DSC measurements (Fig. 2A) showed that the quintuple mutant had an increased \(T_m\) value of 32.9 °C and enhanced thermodynamic stability (\(\Delta C_p\)) of 5.86 kcal/mol at the \(T_m\) value of wild-type PA \(c_{551}\) (Table I). These values were nearly the same as those of HT \(c_{552}\). The quintuple mutant exhibited a large increase in \(\Delta H\) compared with the wild-type PA \(c_{551}\) (Table I), suggesting that the mutant was enthalpically stabilized. In contrast, HT \(c_{552}\) was stabilized by a small \(\Delta S\) rather than by an enthalpic factor (Table I). This is obvious from the heat capacity curve (Fig. 2A), since the peak height and area (nearly representing \(\Delta H_m\)) of HT \(c_{552}\) were smaller than those of the quintuple mutant although their \(T_m\) values were nearly the same. These results suggest that the enhanced \(T_m\) values of HT \(c_{552}\) and the quintuple mutant are mainly due to the five residues (Ala-7, Met-13, Tyr-34, Tyr-43, and Ile-78, numbered as in the quintuple mutant of PA \(c_{551}\)); however, the stabilizing factors differ in the two stable proteins.

Additivity of Thermal Stabilization—We estimated \(\Delta G\) values for the reported PA \(c_{551}\) mutants (1) having F7A/V13M, F34Y/E43Y, and V78I substitutions, respectively, by DSC measurements (data not shown). The estimated values were as follows: F7A/V13M, 2.39; F34Y/E43Y, 2.52; and V78I, 0.82 kcal/mol. The \(\Delta G\) value of the quintuple mutant obtained in this study was almost identical to the value for the hypothetical difference in free energy change, \(\Delta G_{hyp}\), i.e. the sum of the \(\Delta G\) values for the mutant proteins with the F7A/V13M, F34Y/E43Y, and V78I substitutions, respectively (Table I). This indicates that the mutations in each of the three regions contribute in an additive manner to the enhanced overall stability. The three mutated regions in the quintuple mutant do not interact with each other; thus, they may behave independently without nonlocal structural perturbations.

Structure of the Quintuple Mutant Protein—We next determined the solution structure of the quintuple mutant PA \(c_{551}\) protein using 1545 NOE-based distance restraints (comprising 592 intraresidue and intraheme, 300 sequential, 257 medium range, 396 long range including NOEs between the heme and polypeptide chain), supplemented with 102 dihedral and 46 hydrogen bond restraints (Fig. 3A). The best 20 structures for the quintuple mutant satisfied the experimental constraints with small deviations from the idealized covalent geometry (Table II). The stereochemical quality of the 20 structures was determined using PROCHECK-NMR (39). Ignoring all glycines and prolines,
98.5% of the remaining residues fell into the most favored and additional allowed regions of φ and ψ spaces. The average atomic root mean square (r.m.s.) deviations for heavy atoms of residues 3–80 were 0.40 ± 0.06 Å for the main chain atoms and 0.84 ± 0.05 Å for all atoms. These values indicate that the determined structures were well converged and that the restrained energy-minimized structure could be used as a representative for comparison with those of the wild-type PA c551 and HT c552. The main chain folding of the quintuple mutant was similar to those of the wild-type PA c551 and HT c552 (Fig. 3B; backbone r.m.s. deviation values were 0.84 Å for residues 3–80 of the wild-type PA c551 and 0.99 Å for residues 3–78 of HT c552, respectively). This indicates that the introduction of five mutations into PA c551 does not alter the main chain folding.

**Structure Comparison between HT c552 and the Quintuple Mutant**—The structure of the quintuple mutant showed that the side chains of the introduced Ala-7 and Met-13 filled a small cavity found in the wild-type (Fig. 4A). The F7A/V13M mutations in this region also changed the Ile-18 side chain conformation to the favorable gauche minus one (Fig. 4A). The r.m.s. deviation value for the Ala-7, Met-13, Tyr-27, and Trp-77 heavy chain atoms and the residues 5–20, 25–29, and 75–79 main chain atoms was 1.28 Å when these atoms were superimposed on the corresponding atoms of HT c552.

The two introduced Tyr-34 and Tyr-43 aromatic side chains in the quintuple mutant were adjacent, as found in HT c552, and suggested to undergo a hydrophobic interaction and/or π–π interaction with one another (Fig. 4B). The r.m.s. deviation value for the heavy atoms of the introduced Tyr-34 and Tyr-43 and the main chain atoms of residues 34–44 in the quintuple mutant was 0.91 Å when these atoms were superimposed on the corresponding atoms of HT c552. Molecular modeling of PA c551 with the F34Y mutation predicts that the η oxygen atom of the introduced Tyr-34 forms a hydrogen bond with the guanidyl base of Arg-47; this was also indicated by our previous thermodynamic analysis (1). However, it was not clear from the NMR data whether an extra hydrogen bond exists in the quintuple mutant and HT c552; the mutant was not well defined in the NMR structure.

The mutant structure also showed that the introduced Ile-78 filled a cavity around the heme, which was found in the wild-type (Fig. 4C). The r.m.s. deviation value for the Ile-48, Leu-48, Ile-78, and heme heavy atoms and residues 72–80 main chain atoms in the quintuple mutant was 0.91 Å when these atoms were superimposed on the corresponding atoms of HT c552.

These comparisons of side chain interactions in the three mutated regions of the three proteins clearly showed that the regions in the quintuple mutant became more like those in HT c552.

**Difference in Accessible Surface Area**—We further evaluated, using the structural data, the effects of the five mutations in the three regions on the total ASA (accessible surface area). The quintuple mutant and HT c552 in their native states had larger ASA values compared with that of the wild-type PA c551; this was due to the larger polar ASA (ASA pol) value in both cases (Table III). Thus, undefined hydrophilic and polar groups may be more exposed to the solvent. Consequently, the quintuple mutant and HT c552 in the native states exhibited greater negative ΔG_N compared with the wild-type PA c551. Negative ΔG_N values for these two proteins may contribute to the enhanced stability, which is consistent with the results of recent statistical analyses of proteins from thermophiles (40).

**Conclusion**—Our successful design of a mesophilic protein is, to the best of our knowledge, the first example of protein stabilization to the level of a natural thermophilic counterpart by means of limited amino acid substitutions (2, 41, 42). The formation of extra side chain interactions and exposure of hydrophilic and polar groups of the quintuple PA c551 mutant caused the overall elevated stability, which was partly reflected by the increased enthalpy change. The stabilizing strategy for the mutant differed from that in the case of the cold shock protein (43), in which the protein stabilization was mainly achieved through improvement of the electrostatic interaction on the molecular surface.

Our way of carefully comparing the structures of thermo-philic and mesophilic homologous proteins and combining selected mutations is valuable for elucidating the relationship between local side chain interactions and overall protein stability. Now that this has been achieved for the first time, it will be worthwhile exploring the possibility of altering other proteins, especially those of industrial and medical interest, in the same manner.
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