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Influence of Amino Acid Side Chain Packing on Fe−Methionine Coordination in Thermostable Cytochrome *c*

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Proteins isolated from thermophilic organisms have been extensively investigated from both experimental and theoretical points of view to gain an insight into the molecular basis for protein thermostability.[1] Structural comparison among homologous proteins of hyperthermophiles, thermophiles, and mesophiles has contributed significantly to the understanding of the relationship between protein structure and thermostability.[1,2] An increased packing density and hydrophobic core, and the presence of large networks of ion pairs or hydrogen bonds at the protein surface have been proposed to be responsible for thermostability.[1,2] We report herein the results of paramagnetic ^{1}H NMR and optical studies on a homologous cytochrome *c* (cyt *c*) series, which demonstrate that interactions among amino acid side chains in distal regions influence heme axial coordinations in cyt *c*. We propose for the first time that the stability of heme axial coordinations correlates with the overall thermostability of the protein.

Thermophile *Hydrogenobacter thermophilus* cytochrome c_{552} (Ht cyt c_{552}) and mesophile *Pseudomonas aeruginosa* cytochrome c_{551} (Pa cyt c_{551}: structure in Figure 1) are small monoheme-containing electron-transfer proteins, which are composed of 80 and 82 amino acid residues, respectively. The two proteins exhibit high sequence identity (56%),[5] and their main-chain folding is almost identical.[2a] But Ht cyt c_{552} is much more stable as to heat than wild-type Pa cyt c_{551}.[2] A quintuple mutant of Pa cyt c_{551} (F7A/V13M/F34Y/E43Y/V78I), in which amino acid substitutions were selected with reference to the corresponding residues in Ht cyt c_{552}, exhibits thermostability comparable to that of natural Ht cyt c_{552}.[2c] Compared with wild-type Pa cyt c_{551}, the quintuple mutant has a reduced void space, as found in Ht cyt c_{552}. In addition, all the mutated residues are distant from the heme iron so that the heme coordination should not be directly affected by the mutations (Figure 1).

We first examined whether the stability of heme coordination correlates with the overall protein thermostability in Ht cyt c_{552}, wild-type Pa cyt c_{551}, and the quintuple mutant.[6] Figure 2 shows 600 MHz ^{1}H NMR spectra[7] of the oxidized forms of the cyts *c* at various temperatures. Paramagnetically shifted signals arising from heme peripheral methyl and Fe-coordinated methionine protons were observed in downfield and upfield shifted regions of the spectra, respectively. The observation of well-resolved heme methyl proton signals demonstrated that the structural features of the heme active site in Ht cyt c_{552} were intact even at 85 °C (Figure 2A). In contrast to Ht cyt c_{552}, the paramagnetically shifted signals of the oxidized form of wild-type Pa cyt c_{551} disappeared at 75 °C (Figure 2C), indicating that the native conformation around the heme active site was not formed at this temperature. This is consistent with a previous study showing that the overall thermal denaturation of the oxidized form of wild-type Pa cyt c_{551} occurs at around 70 °C.[2b] We also carried out the same measurements for the quintuple mutant possessing overall thermostability comparable to that of Ht cyt c_{552}. Well-resolved signals were clearly observed at 85 °C for the mutant (Figure 2B), indicating that the heme active site was stabilized by the amino acid replacements introduced in this mutant.

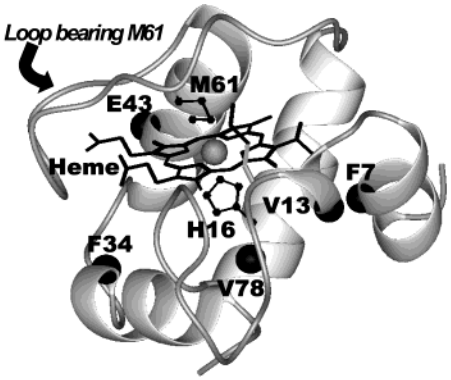

Figure 1. Schematic representation of the structure of Pa cyt c_{551}[3] and the locations of amino acid residues replaced in the quintuple mutant. The polypeptide chain is illustrated as a ribbon model and heme as a stick model. The α carbon atoms of the five mutated residues are denoted by black spheres. The heme prosthetic group is covalently bound to the protein through C12 and C15, and its heme Fe is coordinated to H16 and M61 in Pa cyt c_{551}. The loop bearing the Fe-coordinated M61 acts as a "lid" of the heme active site. MOLMOL[4] was used to draw this figure.

We then examined whether the heme active sites are stabilized by the heme-protein linkage through Fe−methionine coordination in Ht cyt c_{552} and the quintuple mutant. For this purpose, we analyzed the paramagnetically shifted signals for a modified cyts *c*, in which the heme axial methionine was replaced by exogenous CN^-, thus detaching the heme-protein linkage through the Fe−methionine coordination bond. The heme methyl proton shift patterns of the CN^- adducts of all three cyts *c* (top traces in Figure 2) were similar not only to each other but also to that of horse heart cyt *c*.[9] Resolved signals of the CN^- adducts of Ht cyt c_{552} and the quintuple mutant were observed up to 75 °C (data not shown), while those of wild-type Pa cyt c_{551} disappeared at about

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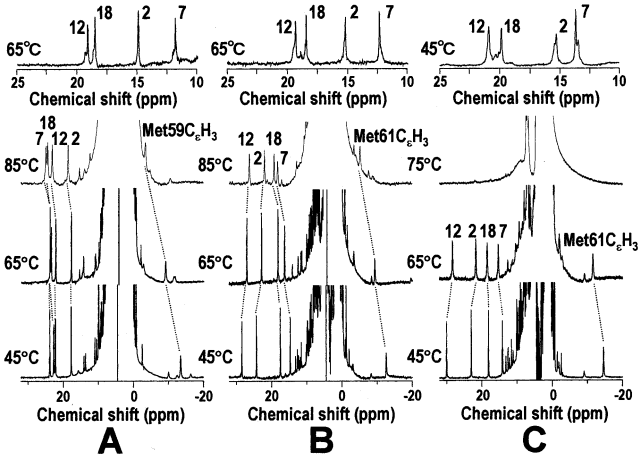

Figure 2. ^{1}H NMR spectra (600 MHz) of the oxidized forms of Ht cyt c_{552} (A), the quintuple mutant (B), and wild-type Pa cyt c_{551} (C) in 90% H_2O/10% D_2O, pH 7.20, at the indicated temperatures. The spectra for wild-type Pa cyt c_{551} were reproducible in the temperature range 5−65 °C, and the protein was soluble at least up to 85 °C. The spectral changes for Ht cyt c_{552} and the quintuple mutant were completely reversible in the temperature range 5−85 °C. The downfield-shifted portions of the 600 MHz ^{1}H NMR spectra of CN^- adducts of the proteins at pH 9.80 and the temperatures are given in the top of each panel. Signal assignments made with standard NMR technique are given with the spectra. The nomenclature of heme approved by IUPAC−IUB Joint Commision[8] was adopted to label heme methyl groups.

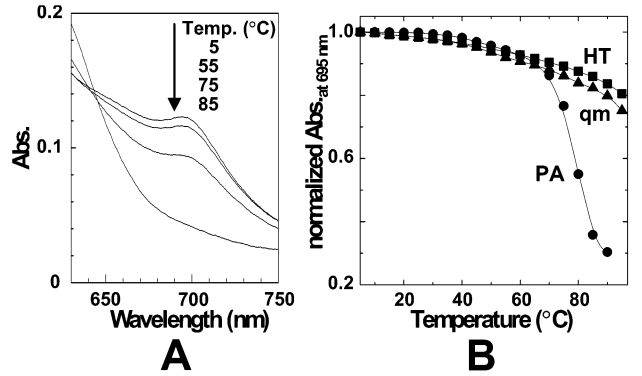

Figure 3. Absorption spectra[11] of the oxidized forms of wild-type Pa cyt c_{551} at the indicated temperatures (A) and the plots of the absorbance at 695 nm of oxidized forms of Ht cyt c_{552} (HT) the quintuple mutant (qm), and wild-type Pa cyt c_{551} (PA) at pH 7.00. The absorbance of the proteins was normalized in such a way that the values at 5 °C equal 1.0.

60 °C. Therefore, the denaturation temperature of the heme active site in the CN^- adduct was largely depressed relative to that of the oxidized form of the corresponding protein. These results indicate that the heme-protein linkage through the Fe−methionine bond contributes significantly to the thermostability of the protein.

The axial methionine is located in a long loop in Pa cyt c_{551}[3] (Figure 1), and a structural change of this loop, triggered by rupture of the Fe−methionine bond, is assumed to initiate overall protein denaturation. Such a hypothesis can be unambiguously verified through the measurements of 695-nm absorption band characteristic of the coordination of methionine sulfur to heme iron in the oxidized forms of cyts *c*.[10] The absorbance at 695 nm for the oxidized form of wild-type Pa cyt c_{551} decreased remarkably with increasing temperature above 70 °C and completely disappeared at about 90 °C (Figure 3A), with the melting point of 78 °C[12] determined as the point of inflection for the plots of the absorbance against temperature (Figure 3B). Thus, the rupture of the Fe−methionine bond in thermal denaturation of this protein was clearly manifested in these spectral changes. On the other hand, the absorbance for the oxidized forms of Ht cyt c_{552} and the quintuple mutant was decreased by only about 30%, and the spectral changes were essentially reversible in the temperature range 5−85 °C. Therefore, structural stability involved in the Fe−methionine coordination in the two proteins can be spectrophotometrically confirmed by these experiments.

Paramagnetic NMR and optical characterization of the heme active sites in the two homologous cyts *c* and the mutant clearly showed that the stability of the Fe−methionine coordination bond correlated well with their overall thermostability. This is manifested by the temperature-resistant appearance of both paramagnetically shifted ^{1}H NMR signals and the 695-nm absorbance due to the Fe−methionine bond. The increased stability of the Fe−methionine bond in the quintuple mutant can be ascribed to reinforcement of the protein interior due to the decreased void space caused by the mutations in remote regions from the heme active site. These findings indicate that alteration in interactions among amino acid side chains in the protein interior influences the stability of Fe−methionine coordination at the heme active site, which in turn affects thermostability of the protein. This leads to a possibility of tuning the Fe^{2+}−Fe^{3+} redox couple of cyt *c* through the polypeptide structure.

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(6) Ht cyt c_{552} was isolated from *H. thermophilus* cells,[5] and wild-type Pa cyt c_{551} and the mutant were prepared as reported previously.[2b,c]

(7) The NMR spectra were recorded on a Bruker Avance 600 FT NMR spectrometer operating at the ^{1}H frequency of 600 MHz. The protein concentration in the samples was about 1 mM in 90% H_2O/ 10% ^2H_2O. Chemical shifts are given in ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate with the residual H^2HO as internal reference.

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(11) The absorption spectra were recorded with Beckman DU 640 spectrophotometer using micro T_m analysis system and micro T_m Cell. The protein concentration was about 0.2 mM in 20 mM phosphate buffer (pH 7.00) in the presence of 10 mM potassium ferricyanide.

(12) The disappearance of the paramagnetically shifted ^{1}H NMR signals of the oxidized form of wild-type Pa cyt c_{551} below 78 °C (Figure 2C) indicated that the rupture of the Fe−methionine coordination bond is not the only requisite for the loss of the NMR signals. The coordination structure as well as electronic nature of heme crucially influences these signals. Detailed studies on conformation and electronic structure of the heme active site at elevated temperatures are in progress.

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