Destabilization of the Heme Region in Mutant Cytochrome c by Replacement of Phe-82 with 3-(Pyren-1-yl)-L-alanine

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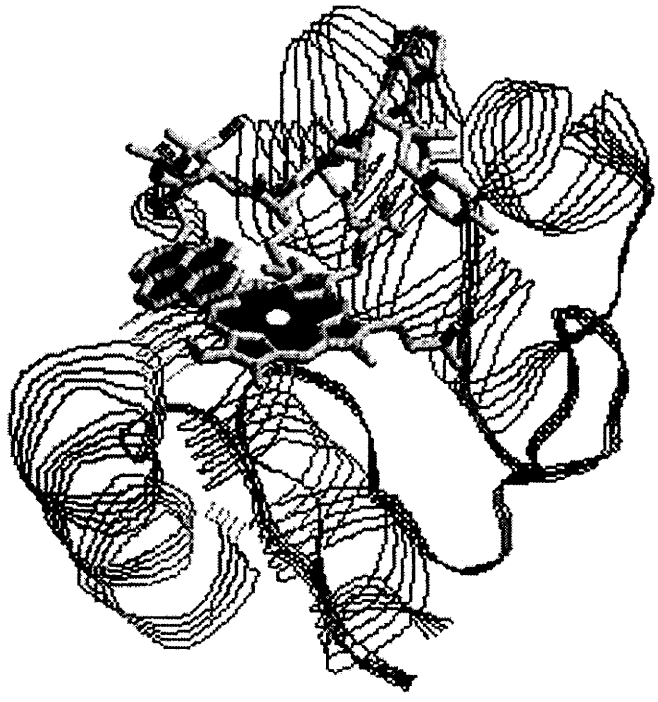
Using a semisynthetic method, mutant cytochromes c, in which 3-(pyren-1-yl)-L-alanine (PyrAla^{82}) or L-phenylglycine (PGL^{82}) replaced a conserved phenylalanine residue (Phe-82) located near the heme, were produced. The PyrAla residue in the reduced state of the mutant cytochrome c was shielded from the access of acrylamide in the aqueous phase, while it is exposed to acrylamide in the oxidized state. The denaturation profile of the mutant protein induced by the addition of GdnHCl was the same as that of the native cytochrome c. Therefore, the hydrophobic heme pocket should retain the native structure in terms of accessibility of acrylamide and GdnHCl. However, thermal denaturation of [PyrAla^{82}]cytochrome c showed that the deformation of the heme region occurred at 54 °C, which was lower than the value of 64 °C for the native one. Thermodynamic analysis revealed that the heme pocket was stabilized by the hydrophobic effect of PyrAla. However, the hydrophobic stabilization is overwhelmed by a large entropy change upon denaturation, resulting in destabilization of the heme region. [PGL^{82}]cyctochrome c, in which the phenyl group is dislocated by one methylene unit from [Phe-82]cytochrome c, was also destabilized in the heme region compared with the native cytochrome c, primarily due to decrease of the hydrophobic effect.

Cytochrome c is a peripheral protein, which has a heme group linked covalently to the polypeptide backbone.† Cytochrome c has been engineered for structural analysis,[1,2] enhancing thermostability[3] and so forth. The tertiary structure of cytochrome c is so flexible that a reversible unfolding was observed in a phospholipid bilayer membrane.[4,5] It is well known that cytochrome c takes a sub-state conformation, which is different from the native or from the fully denatured conformation in the presence of denaturant or in a high pH medium. This state is called a 'molten globule' state, in which cytochrome c retains nearly the same secondary structures as those of native protein, while the tertiary structure is distorted. In our previous investigation, a mutant cytochrome c (PYR-Cytc), in which Phe-82 was replaced with 3-(pyren-1-yl)-L-alanine (PyrAla), was shown to preserve a high content of α-helix, but the structure was distorted (Scheme 1).[6] As a result of the conformational deformation, the mutant cytochrome c possesses a new pathway for electron transfer, 'the second crevice', through which small reductants interact with the heme directly or indirectly through the pyrenyl residue. In order to evaluate the degree of structural distortion induced by the incorporation of an unnatural aromatic amino acid into cytochrome c, the stability of the tertiary structure of the mutant cytochromes c was studied in detail.



Scheme 1 Presentation of PYR-Cytc

Experimental

Materials.—The mutant cytochromes c were prepared by a semisynthetic method as reported previously.[6] The native cytochrome c and the mutant cytochromes c were oxidized by oxygen bubbling into deionized water for 2 h at room temperature and were subsequently lyophilized. All experiments were performed with ferricytochrome c. A stock solution of cytochrome c (4 mg cm^{-3}) in 10 mmol dm^{-3} potassium phosphate buffer (pH 7.2) was prepared and diluted before use. The concentrations of the mutant cytochromes c as well as the native cytochrome c in the oxidized state in 10 mmol dm^{-3} potassium phosphate buffer at 25 °C was determined spectrophotometrically using the molar extinction coefficient of $\varepsilon_{409} = 9.20 \times 10^4$ dm^3 mol^{-1} cm^{-1}.[7]

Other chemicals of the highest grade of purity available (Wako pure chem. Ind., Ltd.) were used without further purification.

Spectroscopy.—Absorption and fluorescence spectra were recorded on a JASCO Ubest-50 UV–VIS spectrophotometer and a Hitachi MPF-4 fluorescence spectrophotometer, respectively, using a cell of 1 cm path length. Circular dichroism spectra were measured by a JASCO J-600 CD spectrophotometer using a cell of 0.1 cm path length. Fluorescence from the PyrAla residue

† *Abbreviations.* CD, circular dichroism; GdnHCl, guanidine hydrochloride; PGL, L-phenylglycine; PGL-Cytc, [PGL^{82}]cytochrome c; PYR-Cytc, [PyrAla^{82}]cytochrome c; PyrAla, 3-(pyren-1-yl)-L-alanine; T_m, midpoint temperature of thermal denaturation.

was measured with excitation at 345 nm. For the fluorescence-quenching experiments, a stock solution of acrylamide (1.0 mol dm^{-3}) was prepared and a small portion of the solution was added to the 1 cm^3 of sample solution in 10 mmol dm^{-3} potassium phosphate buffer (pH 7.2).

Thermal Denaturation.—Thermal denaturation experiments were carried out on the CD or UV spectrometer equipped with a water jacket to control the temperature of the sample solution. The temperature was raised with a rate of 1 degree min^{-1} from 10 to 80 °C. The signal strength at 222 nm in CD spectra and at 692 nm in UV spectra was monitored in 10 mmol dm^{-3} potassium phosphate buffer (pH 7.2) of 1.1×10^{-6} mol dm^{-3} cytochromes c.

Melting curves were analysed by using a linear least squares program. The thermal unfolding equilibrium constant at temperature T, $K(T)$, in the cooperative transition region was calculated according to eqn. (1), where $A_{692}(T)$ represents the

$$K(T) = \frac{A_N - A_{692}(T)}{A_{692}(T) - A_D} \quad (1)$$

absorbance at 692 nm at temperature T, A_N and A_D the absorbance in the native state and the denatured state, respectively. The van't Hoff parameters ΔH_0 and ΔS_0 were obtained from the slope $(-\Delta H_0/R)$ and intercept $(\Delta S_0/R)$, respectively, of the plot of ln $K(T)$ against $1/T$. T_m, the midpoint of the denaturation, was calculated from $T_m = \Delta H_0/\Delta S_0$ where $\Delta G_0 = 0$ kcal mol^{-1}. The difference of free-energy change in the thermal unfolding of the wild-type protein and the mutant protein, $\Delta\Delta G_0$, was calculated by the eqn. (2), where the

$$\Delta\Delta G_0 = \frac{\Delta H_0^w \Delta T}{T_m^w} \quad (2)$$

superscript w represents the quantity for the native cytochrome c and ΔT represents the difference of T_m between the two types of proteins.[8]

Denaturation with Guanidine Hydrochloride (GdnHCl).—Unfolding of cytochrome c by the addition of GdnHCl was monitored by the change of the Cotton effect at 222 nm in CD spectra.[9] Measurements were done at 25 °C within approximately 10 min after mixing the protein with GdnHCl. The signal at 222 nm was measured at least for 2 min and averaged by using a personal computer.

The denaturation curves obtained were analysed by the standard procedure[10] according to Schellman's two-state model for the equilibrium N↔U, in which N and U denote the native state and the denatured state, respectively.[10]

The equilibrium constant, K_u, was calculated from the fractions of unfolded and folded proteins. K_u as a function of [GdnHCl] was analysed by the equation, $\Delta G_u = \Delta G_u^\circ - m$ [GdnHCl] to obtain the parameter ΔG_u° (free energy of unfolding in the absence of denaturant), m (cooperativity parameter of unfolding) and C_m ([GdnHCl] necessary to denature 50% of protein).[11]

Denaturation by pH *Increase.*[12]—The pH of a solution of cytochrome c (3 cm^3) was adjusted by adding a 0.1 mol dm^{-3} NaOH solution in an optical cuvette at 25 °C, with stirring. After each addition of NaOH solution, the absorbance at 692 nm was monitored until it reached a stable value.

The change in absorbance at 692 nm of the native cytochrome c and the mutant cytochrome c as a function of pH

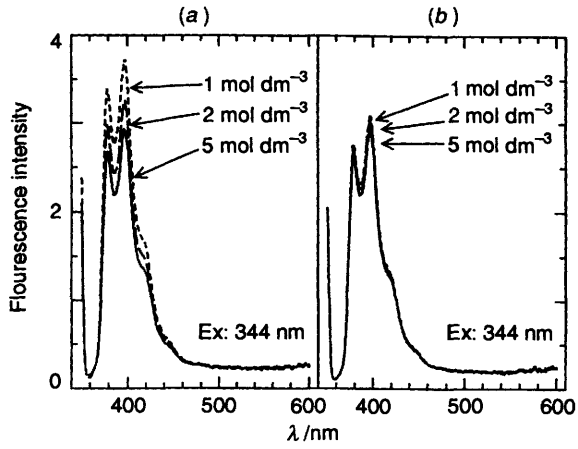


Fig. 1 Fluorescence quenching of oxidized and reduced form of PYR-Cytc. (*a*) Oxidized form. Fluorescence spectra of ferricytochrome c in the presence of 1, 2 and 5 mmol dm^{-3} of acrylamide. (*b*) Reduced form. Fluorescence spectra of ferrocytochrome c in the presence of acrylamide. PYR-Cytc was completely reduced by adding 1% (v/v) of 2-mercaptoethanol followed by standing for 24 h at room temp. in the dark and subsequent gel permeation chromatography on Sephadex G-25 (fine grade) column with potassium phosphate buffer solution (pH 7.2) as eluent. The excitation wavelength was 344 nm.

was analysed by using eqn. (3),[13] where A_{692}^N and A_{692}^D represent the absorbance of the native and the denatured

$$pK_a = -\log[(A_{692}^N - A_{692})/(A_{692} - A_{692}^D)] + n\text{pH} \quad (3)$$

(alkaline form) protein at 692 nm, respectively, and m is the number of deprotonations of the protein responsible for the absorbance change at 692 nm. The apparent pK_a of each protein for the equilibrium between the two states was determined by non-linear least-squares fitting of a $\log[(A_{692} - A_{692}^N)/(A_{692}^D - A_{692}^N)]$ *vs.* pH plot to the theoretical equation, $\log[(A_{692} - A_{692}^N)/(A_{692}^D - A_{692}^N)] = A_{692}^N/(1 + 10^{m\text{pH} - pK_a})$. A free-energy difference between the native and the mutant cytochrome c in the alkaline form, $\Delta\Delta G_{alk}$ was calculated by the equation $\Delta\Delta G_{alk} = -2.3RT\Delta pK$.

Results and Discussion

Fluorescence Quenching of the Pyrenyl Residue with Acrylamide.—It has been shown that the α-helix content of PYR-Cytc was decreased and the heme region was distorted as evidenced by the broadened Soret band.[6] Therefore, it was expected that the incorporation of the bulky pyrenyl group might result in loosening of the highly ordered structure due to steric effects and hydrophobic interactions between the pyrenyl group and the hydrophobic heme pocket. However, the CD spectrum of the far-UV region showed that the mutant retains a certain kind of ordered structure including a high content of α-helices.[6]

The environment of the pyrenyl residue in PYR-Cytc was studied by fluorescence quenching using acrylamide. In the case of native cytochrome c, the phenyl group of phenylalanine residue at the position 82 reversibly changes the location depending on the redox state of cytochrome c. The phenyl group is packed in the hydrophobic heme pocket in ferrocytochrome c, while it is transferred to the protein surface, accessible to an aqueous medium, upon one-electron oxidation to ferricytochrome c.[14–17] The fluorescence intensity of the pyrenyl group in the oxidized form of mutant cytochrome c was quenched by the addition of water-soluble quencher, acrylamide (Fig. 1). On the other hand, no quenching was observed in the reduced form of the mutant cytochrome c.

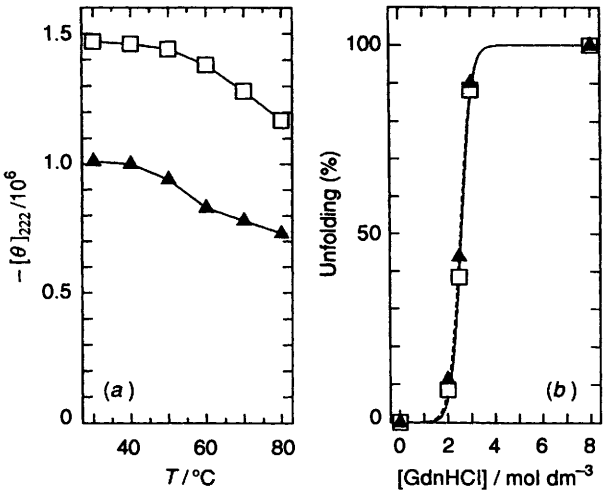


Fig. 2 Secondary structure stability of the native cytochrome c and PYR-Cytc. (*a*) Thermostability of the cytochromes c was monitored by CD at the wavelength 222 nm. Square symbols represent the native cytochrome c, and triangles PYR-Cytc. (*b*) Chemical unfolding of cytochromes c by the addition of GdnHCl as denaturant. Smooth lines are fitting curves calculated by a non-linear least squares program. Square and triangle symbols represent the native cytochrome c and PYR-Cytc, respectively.

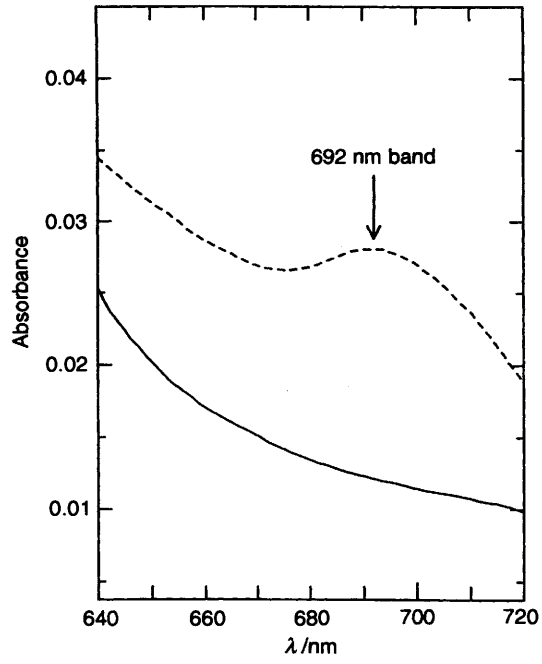


Fig. 3 UV spectra in the far IR region of PYR-Cytc. (– – –) PYR-Cytc of 40 μmol dm^{-3} in 10 mmol dm^{-3} potassium phosphate buffer solution at pH 6.9. (——) PYR-Cytc under the same conditions except pH = 11.0.

This result means that the conformational change in the heme region of the mutant cytochrome c occurs depending on the redox state of the heme and takes place similarly to the native cytochrome c. It is interesting that the bulky pyrenyl group is fully buried in the hydrophobic pocket of the mutant protein in the reduced state.

Denaturation Investigated by CD.—The stability of the tertiary structure of the mutant cytochrome c was analysed on the basis of the change of CD spectra induced by raising the temperature or adding GdnHCl. Fig. 2. shows the thermal stability and chemical unfolding of cytochrome c. Both experiments were monitored by the change of θ_{222}, which reflects the extent of denaturation of the helical region of cytochrome c. The native cytochrome c and PYR-Cytc similarly changed the intensity upon raising the temperature [Fig. 2(*a*)]. However, a minor conformational change was found at about 55 °C only in the case of PYR-Cytc, suggesting a structural perturbation of the heme environment.[18] It has been reported that the excess specific heat *versus* temperature curve for ferricytochrome c in an aqueous buffer solution (pH 7.4) showed a major endothermic transition at 81 °C, which is preceded by a minor endotherm centred around 64 °C. While the transition at 81 °C is ascribed to a major conformational change of the polypeptide backbone, the minor transition is considered to be due to loosening of the protein structure in the vicinity of heme crevice.[18] Thus, the structural perturbation in the heme region of PYR-Cytc might become significant compared with the native cytochrome c.

On the other hand, in the chemical unfolding by GdnHCl, the native cytochrome c and PYR-Cytc behaved in exactly the same way [Fig. 2(*b*)]. The curves were fitted by non-linear least squares analysis to determine thermodynamic parameters (Table 1). Both parameters, $\Delta G°$ and m, are nearly the same for the native cytochrome c and PYR-Cytc. The result suggests that PYR-Cytc is as stable as the native cytochrome c in terms of hydrogen-bonding effects, since GdnHCl breaks hydrogen bonds by interaction with peptide bonds. The minor transition at around 55 °C in the thermal denaturation of the mutant cytochrome c was not observed in Fig. 2(*b*). It is considered that GdnHCl, at low concentrations, interacted only with the surface of the globular protein and unfolding proceeded gradually from the surface to the bulk of the protein molecule with increasing concentration of GdnHCl. Therefore, the preliminary unfolding of the heme environment could not have been detected by this procedure.

Table 1 Thermodynamic parameters[a] for the conformational change of the protein backbone

	Native	Mutant	Reported[b]
$\Delta G°$/kcal mol^{-1} [a]	6.47	6.21	6.2
m/kcal mol^{-2} [a]	4.37	4.27	2.25

[a] Calculated from a θ_{222} *vs.* GdnHCl plot. [b] In 20 mmol dm^{-3} phosphate buffer solution (pH 7.0) at 25 °C.[19]

Denaturation Monitored by Absorbance at 692 nm.—One of the most informative absorption bands on the heme environment is a weak charge-transfer band at 692 nm, which arises from the interaction of the heme ferric iron and the sixth coordination ligand, the thioether of methionine 80.[20] Though the extinction coefficient is relatively small, it is sensitive to pH- or temperature-induced perturbation of the heme environment. The mutant cytochromes c also show a weak absorption at 692 nm, which disappears completely at pH 11.0 (Fig. 3). Therefore, this absorption is ascribed to the Fe–S bonding and reflects the stability of the heme environment.

The changes of absorbance at 692 nm of the native cytochrome c, PYR-Cytc and the fully N^{ε}-amidinated cytochrome c with increasing pH are shown in Fig. 4. pK_a values were obtained by curve fitting of the data and are shown in Table 2, with the numbers of deprotonation of the protein for pH-induced denaturation, m. The pK_a of the fully N^{ε}-amidinated cytochrome c increased by 3.8 pH units compared with that of the native cytochrome c. This shift is qualitatively explained by the higher pK_a value of the amidinated group than the primary amino group of lysine. The pK_a of PYR-Cytc was calculated to be 8.8, which is slightly higher than the 8.3 of the native cytochrome c. The increase of 0.5 pH unit corresponds to 0.65

Table 2 Thermodynamic parameters[a] for conformational change of the heme environment

Transition parameters[a]	Cytochrome c			
	Native	Mutant	Amd-Cytc[b]	Reported[c]
pK_a	8.3	8.8	12	9.1[d]
m	0.91	0.95	1.3	—
$\Delta\Delta G_{alk}$/kcal mol^{-1}	0.0	−0.65	−5.1	—

[a] Determined by absorption change at 692 nm upon varying pH. [b] Fully ε-amidinated cytochrome c. [c] In 10 mmol dm^{-3} sodium cacodylate, 10 mmol dm^{-3} sodium chloride (pH 7.0).[22] [d] In 0.1 mol dm^{-3} NaCl, 0.1 mmol dm^{-3} protein, at 25 °C.[21]

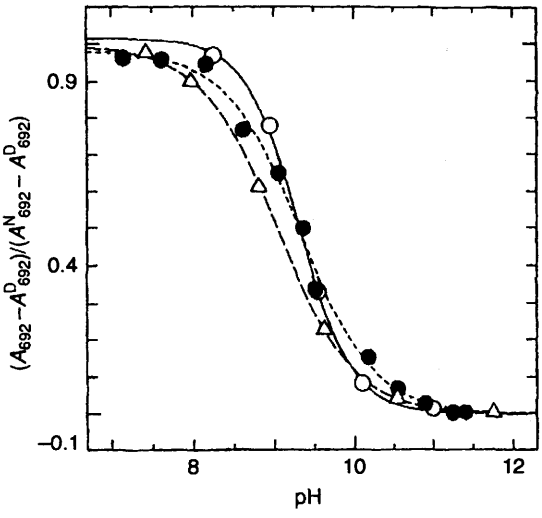


Fig. 4 Alkaline transition of the native cytochrome c (●), PYR-Cytc (△) and fully N^{ε}-amidinated cytochrome c (○). The protein concentrations were 40 μmol dm^3. The data were subjected to a curve-fitting program and the results are shown by three types of lines. The relation coefficients are in the range of 0.9978–0.9999. The titration was monitored by the absorbance change at 692 nm at 25 °C.

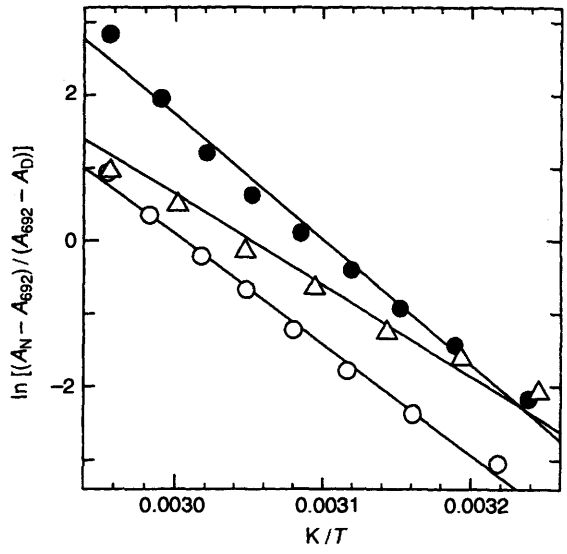


Fig. 5 Thermostability of the native cytochrome c (○), PYR-Cytc (●) and PGL-Cytc (△). Sample solutions were prepared in 10 mmol dm^{-3} potassium phosphate buffer solution (pH 7.2). The denatured/native ratio, K, is plotted against the reciprocal of absolute temperature. The intercept at ln K = 0.0 gives the T_m.

kcal mol^{-1} stabilization of the heme iron–methionine bond and is smaller than that reported for F82I and F82L mutants of yeast iso-1-cytochrome c, which showed a 1.3 pH unit increase.[21]

The disappearance of the 692 nm band at high pH is generally interpreted to be a result of the replacement of the normal methionine ligand with a deprotonated form of a lysine residue due to conformational change in the heme crevice.[23] This explanation is also applicable to PYR-Cytc, because m values determined from the titration curves are approximately unity, suggesting that the structure change is accompanied by a one proton transfer. The increase of pK_a induced by replacement of Phe-82 with PyrAla was not so large as that of F82I or F82L mutants. One possible explanation for the pK_a shift is that the access of the deprotonated amino group of lysine to the coordination site of the methionine with the heme group is hindered by the bulky pyrenyl group in the heme pocket.

Thermal Denaturation of the Heme Region.—Thermal denaturation of the native and the mutant cytochromes c was monitored by the change of absorbance at 692 nm. The Arrhenius plots of the thermal denaturation are shown in Fig. 5 and the thermodynamic parameters calculated from the plots are summarized in Table 3. The free-energy change for the conformational change of the heme environment, $\Delta G°$, reveals that PYR-Cytc and PGL-Cytc are less stable than the native cytochrome c by 0.7 and 1.5 kcal mol^{-1}, respectively. It should be noted that the instability of the heme region of PYR-Cytc is due to the large entropy change, $\Delta S°$. The enthalpy term of PYR-Cytc contributes to the conformational stability to a larger extent than that of the native cytochrome c. The large entropy change of the mutant cytochrome c might be explained by the lowered flexibility of the heme region, which is consistent with the crowdedness of hydrophobic heme pocket as described before. The melting temperature of 54 °C coincides well with the minor thermal transition at about 55 °C observed in the experiment of thermal denaturation [Fig. 2(*a*)]. Table 3 also shows thermodynamic parameters of PGL-Cytc. In the PGL-Cytc, one β-methylene of Phe-82 was lost, resulting in a decrease of the melting point (T_m = 55 °C) as low as that of PYR-Cytc. In this case, a large difference in the enthalpy change ($\Delta\Delta H_0$ = −5 kcal mol^{-1}) was observed, whilst the entropy change was comparable to that of native cytochrome c. These results suggest that the aromatic group of Phe-82 of the native cytochrome c contributes to the stability of the heme region probably due to a hydrophobic effect. Introduction of a large aromatic group should not impair the hydrophobic effect on the tertiary structure, but may destabilize the structure by putting high constraint on the structure.

Conclusions

The conformational stability of proteins is usually low (5–20 kcal mol^{-1}) and varies by the influence of the opposing factors of the hydrophobic effect and the entropy effect on the folded state.[24] Hydrophobic amino acid residues generally contribute to the stability of the hydrophobic region as shown by the conserved tryptophan residue of cytochrome c acting as a

Table 3 Thermodynamic parameters[a] for conformational change of the heme environment

Transition parameters[a]	Cytochrome c			
	Native	Mutant	Mutant[b]	Reported[c]
ΔS_0/cal mol^{-1} K^{-1}	88	104	76	62
ΔH_0/kcal mol^{-1}	30	34	25	21.1
ΔG_0/kcal mol^{-1}	3.7	3.0	2.2	2.45
T_m/°C	68	54	55	65

[a] Determined by absorption change at 692 nm upon varying temperature. [b] Mutant cytochrome c substituted with L-phenylglycine at position 82. [c] In 10 mmol dm^{-3} sodium cacodylate, 10 mol dm^{-3} sodium chloride (pH 7.0).[22]

conformation-stabilizing unit.[12] The replacement of Phe-82 with PyrAla[82] of cytochrome c strengthened the hydrophobic effect as shown in the increase of ΔH for thermal denaturation, but destabilized the overall conformation due to the large ΔS for denaturation.

Replacement of an amino acid residue of a protein may significantly affect its stability, but substitution of a conserved amino acid residue of cytochrome c does not always result in destabilization of the protein. It has been reported that the midpoints of GdnHCl denaturation for the mutant proteins (K32L, K32Q) are a little smaller than the wild type.[24] It has also been reported that hydrophobic interactions are the main factor for increasing stability of the Ile52 mutant of yeast iso-1-cytochrome c.[25] In the present study, the conserved Phe-82 residue was replaced by a PyrAla residue. The overall backbone structure was not drastically altered, but the conformational change took place in the hydrophobic heme pocket. These results suggest that the mutant cytochrome c takes a kind of 'molten globule' structure. It is interesting, however, that a bulky PyrAla residue is accommodated in the hydrophobic heme pocket in the reduced state of the mutant cytochrome c. In the oxidated state, a significant deformation at the heme region is accompanied by exposure of the PyrAla residue to the medium. The structural change might yield another heme crevice, which acts as an alternative pathway for electron transfer as reported in the previous paper.[6]

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