Semisynthesis of Mutant Cytochromes *c* Replaced by Non-natural Aromatic Amino Acid at Phe^{82} and Photoregulation of Reduction Reaction with 2-Mercaptoethanol

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Mutant cytochromes *c*, PGL-Cytc and PYR-Cytc, where Phe^{82} was replaced respectively with a non-natural amino acid, *N*-phenylglycine (pheGly) and 3-(pyren-1-yl)-L-alanine (pyrAla), were synthesized by a semisynthetic method. CD measurements revealed that PGL-Cytc nearly retained the tertiary structure of the native cytochrome *c*, whilst PYR-Cytc did not. The reduction of PGL-Cytc by 2-mercaptoethanol was retarded compared with that of the native cytochrome *c*. On the other hand, PYR-Cytc was easily reduced by 2-mercaptoethanol, probably due to structural changes around the haem moiety and interaction of the pyrenyl group with the haem. Excitation of the pyrenyl group of PYR-Cytc upon UV irradiation suppressed the reduction greatly. This is ascribed to the appearance of oxidation from iron(II) to iron(III) haem through photosensitization of the pyrenyl group.

Cytochrome *c* is a haem protein, which is involved in electron-transfer reactions of the respiratory chain in cell membranes such as the mitochondrial inner membrane. It is known that the arrangement of amino acid residues around the haem moiety is crucial for the electron-transfer ability (refs. 1-4 and references therein). The molecular mechanism for the electron transfer over a long distance is still controversial,[5,6] but the polypeptide matrix should play an important role in providing a passage for the partner proteins.[7]

Phe^{82} is an evolutionarily conserved residue within the interfacial region that has long been believed to be important for anchoring cytochrome *c* to its redox partners. Recently, it was suggested that the Phe^{82} is required not for electron-transfer activity but for correct binding between cytochrome *c* and cytochrome *c* oxidase.[8] However, changing only this amino acid residue in yeast iso-1-cytochrome *c* produces nearly as large an effect on the triplet decay rate as does the use of cytochrome *c* (*Drosophila melanogaster*), which differs from yeast iso-1-cytochrome *c* in 40% of its amino acid residues.[9] Since Phe^{82} is located near the haem moiety, it has been postulated that the orbital overlap and the oxidation–reduction-potential difference between the haem and the aromatic side chains should be influential in the early process of electron transfer of the cytochrome *c*.[10]

A useful way to investigate the role of specific amino acid residues of a protein is to study the activity of the protein analogues that are substituted at a specific site.[11] A relationship between the nature of the axial ligand of the haem and the redox potential of mutant cytochromes *c* that are replaced at Met^{80} by natural or non-natural amino acids has been discussed.[12,13] Although protein engineering has been employed for fundamental studies of protein structure–function relations, it has been little applied for developing novel functions of the protein.[14,15] In the present investigation, Phe^{82} of cytochrome *c* was replaced with *N*-phenylglycine (pheGly) or pyrenylalanine (pyrAla), in order to correlate the electron transport with the spatial arrangement of the large aromatic side chain at position 82. Furthermore, it was expected that the electron transfer of the mutant containing pyrAla would be regulated upon irradiation with light, because the oxidation–reduction potential of the pyrenyl group is changed by photoexcitation.[16]

We report here the results of experiments that show the activity change in pyrAla- or pheGly-substituted cytochrome *c* mutant (which we will call PYR-Cytc and PGL-Cytc, respectively) in comparison with native cytochrome *c*, and the photosensitivity of PYR-Cytc activity.

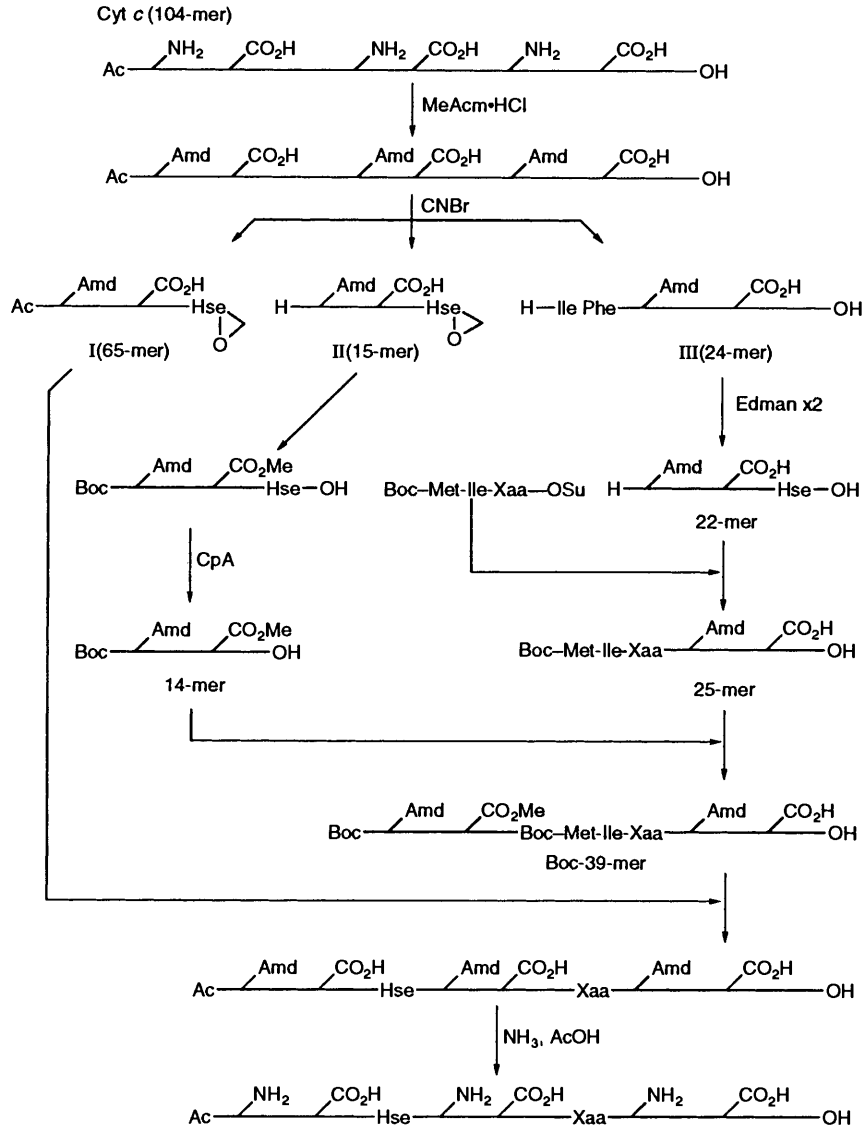
Experimental

Materials.—Horse heart cytochrome *c* (Type VI), and carboxypeptidase A (diisopropylphosphorofluoridate-treated) were obtained from Sigma Chemical Co., St. Louis, USA. *N*-(*tert*-Butoxycarbonyl)methionine hydroxysuccinimide ester (Boc-Met-OSu) and *N*-(*tert*-butoxycarbonyl)isoleucine (Boc-Ile-OH) were obtained from Kokusan Chemical Works Ltd., Tokyo, Japan. Methyl acetimidate hydrochloride was prepared by the method reported by Hunter and Ludwig.[17] Optically pure L-pyrenylalanine was prepared by the method of Egusa *et al.*[18] Other chemicals used in this work were commercially available, highest purity products.

The mutant cytochromes *c* were prepared by using a semisynthetic method as shown in Scheme 1. The preparation was as follows.

Horse heart cytochrome *c* (100 mg) was first subjected to gel filtration on a Sephadex G-50 column to remove polymeric substances. Acetimidylation of the cytochrome *c* was carried out by the method of C. J. A. Wallace.[19] Acetimidyl-cytochrome *c* was obtained as a red powder (95 mg) after gel chromatography. Amino group analysis showed that more than 95% of ε-amino groups were blocked.

The protected cytochrome *c* was split into three fragments I (1–65), II (66–80), III (81–104) by the established method.[20] A 30-fold excess of CNBr over methionine residues was added to a 0.8 mmol dm^{-3} solution of acetimidyl-cytochrome *c* in 70% aq. formic acid. The reaction was performed at 20 °C in the dark for 24 h. The mixture was then lyophilized and was redissolved in 7% (v/v) aq. formic acid, and subjected to gel filtration on a Sephadex G-50 (super-fine grade) column (4.0 × 55 cm). Fragment I lactone was purified by removal of the unreactive derivative of the fragment that had been hydrolysed at the carboxy terminal homoserine lactone (yield 57 mg).[21] Fragment II was dissolved in 0.01 mol dm^{-3} aq. sodium phosphate buffer containing 4 mol dm^{-3} urea (pH 6.9; 10 cm^3) and applied to a DEAE-Sephadex column (1.5 × 30 cm), with a linear sodium phosphate gradient (0.01–0.05 mol dm^{-3}) at a flow rate of 25 cm^3 h^{-1} (yield 9 mg).[21] HPLC analysis showed a single peak of 66–80 fragment. Fragment III was purified on a CM-Sephadex



Scheme 1 Semisynthesis of mutant cytochromes *c*

column (1.5 × 35 cm), with a linear sodium phosphate gradient (0.01–0.15 mol dm^{-3}) at a flow rate of 25 cm^3 h^{-1} (yield 21 mg).[21] HPLC analysis showed a single peak for the 81–104 fragment.

Two cycles of the Edman reaction[22] were carried out to split off the N-terminal residues Ile^{81} and Phe^{82} from fragment III. The stepwise degradation of the fragment was monitored by TLC analysis [R_f = 0.72 of phenylthiohydantoin (PTH)-Ile for the first cycle, and R_f = 0.68 of PTH-Phe for the second cycle]. After two cycles, the degraded peptide was purified by gel filtration on a Sephadex G-50 column in 7% formic acid and desalted by a Sephadex G-25 column. The required fraction was collected as a powder (5 mg) after lyophilization.

N^{α}-Boc-N^{72},N^{73}-diacetimidyl-des-Hse^{80}-fragment II O^{66}, O^{69}-dimethyl ester was prepared as described in the literature[23] with several modifications. Fragment II O^{66},O^{69}-dimethyl ester was prepared by direct esterification of fragment II with 5% methanolic HCl. The α-amino group was protected by treatment of a dimethylformamide (DMF) solution of the peptide ester with a 250-fold molar excess of di-*tert*-butyl dicarbonate, instead of *tert*-butyl azidoformate. The C-terminal Hse^{80} lactone was selectively hydrolysed in 2% aq. triethyl ammonium hydrogen carbonate (pH 9.0). The reaction mixture was adjusted to neutrality (measured by a pH meter) with 1 mol dm^{-3} acetic acid and the solution was lyophilized. The removal of C-terminal residue Hse^{80} was achieved by using carboxypeptidase A (CpA) (50 units cm^{-3}) in 2 mol dm^{-3} aq. ammonium hydrogen carbonate buffer (pH 7.5).[24] The reaction mixture was applied directly to a Sephadex LH-20 column (2.0 × 100 cm) in chloroform–methanol (1:9, v/v). The collected fraction was concentrated.

The tripeptide IV (80–82) was prepared by a conventional method using dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (HOSu) as coupling reagents. Fragment (83–104) was coupled with Boc-tripeptide (80–82) *N*-hydroxysuccinimide ester, Boc-Met-Ile-pyrAla-OSu or Boc-Met-IlepheGly-OSu. The coupling reaction was performed in DMF solution, at a peptide concentration of 1 mmol dm^{-3}, by using a 10–20-fold molar excess of active ester. The reaction was allowed to continue for 2 days at 20 °C. The product peptide was dried over silica gel under high vacuum and treated with anhydrous trifluoroacetic acid (TFA) (40 °C for 60 min, peptide

concentration 5 mmol dm^{-3}). The product was purified by gel filtration on a Sephadex G-50 column with 7% formic acid as eluent (yield 5 mg).

The derivative of the fragment (66–79) was converted into its *N*-hydroxysuccinimide ester and was treated with the fragment (80–104). Coupling was performed in DMF solution, at a peptide concentration of 1 mmol dm^{-3}, by using a 10–20-fold molar excess of active ester. The reaction was allowed to continue for 2 days at 20 °C. The Boc group was removed by treatment with anhydrous TFA at 40 °C for 1 h (peptide concentration 1 mg cm^{-3}). After the treatment, the product was recovered by precipitation with 4-fold volumes of cold diethyl ether. The precipitate was washed once with cold diethyl ether and was dried over silica gel (yield 4 mg). The methyl groups were removed from purified fragment (66–104) by suspension at a concentration of 0.5 mg cm^{-3}, in triethylamine–water (1 : 49, v/v) which had been adjusted to pH 10.5 (measured by a pH meter) (at 20 °C) with 1 mol dm^{-3} acetic acid. After 24 h at 20 °C, the pH was adjusted to 7 with 1 mol dm^{-3} acetic acid, and the resulting solution was lyophilized (yield 4 mg).

The technique of Corradin and Harbury[20] or its adaptation by Wallace and Rose[25] was used in the combination of the haem and non-haem fragments. The lactone of fragment I and the fragment (66–104) of horse cytochrome *c* form a complex in neutral aqueous buffers, in which the missing 65–66 peptide bond is re-formed by aminolysis of C-terminal Hse^{65} lactone when the complex is maintained in a reducing state. Sufficient 2-mercaptoethanol was added to ensure complete reduction of the degassed mixture, which was then sealed to exclude air completely. After 24 h in the reducing state, the mixture was purified by gel filtration[26] (yield 1.2 mg). All the cytochrome *c* analogues prepared contain a homoserine residue at the position 65. This substitution does not significantly affect the chemical or physical properties of cytochrome *c*.[20,27] Lyophilized *N*-amidinated products were deprotected by treatment with aq. NH_4OAc buffer (1 mol dm^{-3}, pH 11.5) at 20 °C in the dark.[28] After 18 h, the solution was subjected directly to a Sephadex G-25 fine column to remove low-molecular mass salts. Haem-containing fractions were collected and lyophilized.

The lyophilized semisynthetic mutants of cytochromes *c* were renatured by the method reported by Babul *et al.*[29] The lyophilized product was treated with 8 mol dm^{-3} urea in aq. potassium phosphate buffer (0.1 mol dm^{-3}, pH 7.0), and then freed from urea on a gel-filtration column of Sephadex G-25 equilibrated with the potassium phosphate buffer. This treatment can renature proteins that might have been denatured by the freeze-drying procedure from 7% formic acid.

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 7.5% separation gel with a 4% stacking gel. An aliquot of the semisynthesized protein, after separation on a Sephadex G-25 column, was mixed with a buffer solution [50 mmol dm^{-3} tris(hydroxymethyl)aminomethane (Tris) (pH 6.9), 10% glycerol, 0.05% Bromophenol Blue, 10% SDS, 5% 2-mercaptoethanol]. The sample was applied to the SDS-polyacrylamide gel after heating the sample at 95 °C for 5 min. The gel was stained with Coomassie Brilliant Blue R-250. PYR-Cytc showed a band at a position nearly the same as that of native cytochrome *c*, indicating that the mutant protein had virtually the same molecular mass as did the native one. A minor band, whose intensity was less than 5%, was detected, and was probably due to the dimer.

Measurements.—UV and visible absorption spectra were recorded on a JASCO Ubest-50 UV–VIS spectrophotometer with a cell of 1 cm path length. The concentration of proteins used in this study was determined by the intensity of the appropriate absorption maximum on the basis of molar extinction constants [ε_{550} (reduced) = 2.95×10^4 dm^3 mol^{-1} cm^{-1} and ε_{409} (oxidized) = 1.10×10^5 dm^3 mol^{-1} cm^{-1} for the haem-containing fragments, and $\varepsilon_{406} = 1.27 \times 10^5$ dm^3 mol^{-1} cm^{-1} for the iron(III) haem fragment I and $\varepsilon_{276} = 9.89 \times 10^3$ dm^3 mol^{-1} cm^{-1} for the non-haem fragment (66–104)]. CD spectra were recorded on a JASCO J-600 CD spectrophotometer with a cell of 0.1 cm path length.

High-performance liquid chromatography (HPLC) was performed on a JASCO LC-800 system. A 5C18 (4.6 × 150 mm) Cosmosil 390-46 column (Nacalai Tesque, Inc., Kyoto, Japan) was used at a flow rate of 1 cm^3 min^{-1}. Solvent A was prepared by adding TFA (HPLC-grade, 1 cm^3) to filtered deionized water (1 dm^3). Solvent B was acetonitrile (HPLC grade, Wako) with 0.1% (v/v) TFA.

Protein concentration was determined by Lowry's method.[30] Free amino groups were determined by the method of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-assay.[31]

The electron-transfer activities of the mutant cytochromes *c* were evaluated by measuring the rate of reduction with 2-mercaptoethanol in aqueous solution. The absorbance at 415 nm assigned to the Soret band in the reduced form was monitored with the addition of 2-mercaptoethanol. The logarithm of absorbance, $\log[A_{415}(0) - A_{415}(t)]$, was plotted against reaction time, and kinetic constants were determined by least-squares fitting analysis.

Results and Discussion

Semisynthesis.—The technique of protein semisynthesis, in which fragments of a protein are condensed by chemical methods, has been established and employed by many authors.[32–39] The use of protein fragments produced by treatment with cyanogen bromide (CNBr) is important because it promises a spontaneous condensation of the fragments formed by aminolysis of the C-terminal homoserine lactone.[27,40,41]

Horse heart cytochrome *c* was amidinated to block primary ε-amino groups and was subjected to treatment with CNBr. CNBr cleaves the protected cytochrome *c* at the two methionine residues to give three fragments corresponding to residues 1–65, 66–80, and 81–104. The fragment III containing Phe^{82} was subjected to Edman degradation. The non-natural amino acids were incorporated by an active-ester method. The fragment was recombined with the other two fragments, and finally the protecting groups were removed to obtain mutant cytochromes *c*.

Protein Structure.—Fig. 1. shows circular dichroism (CD) spectra of mutant cytochromes *c* at the Soret band. Almost identical spectra were observed for native cytochrome *c* and PGL-Cytc, but that of PYR-Cytc is somewhat different from the other two. A positive signal at $\lambda \sim 340$ nm should overlap in the spectrum of PYR-Cytc with the ^1La band of the pyrAla residue. The UV-visible spectrum of PGL-Cytc was virtually identical with the spectrum of native cytochrome *c* in the porphyrin absorption region: Soret band at 408 nm (1.1×10^5 dm^3 mol^{-1} cm^{-1}); Q band at 528 nm (1.0×10^4 dm^3 mol^{-1} cm^{-1}) (Fig. 2). These results indicate that substitution of pheGly for Phe has little effect on the spatial arrangement of aromatic side chains around the haem, and the redox properties of PGL-Cytc and native cytochrome *c* might be nearly identical.

However, the Soret band of PYR-Cytc at 410 nm was broadened, reflecting relocation of aromatic side chains around the haem. UV-visible spectra showed a slight red shift at the Soret band by 2 nm, and some broadening was observed also in the Q-band region (550 nm). This small shift is ascribed to interactions of the haem with the pyrenyl group at position 82

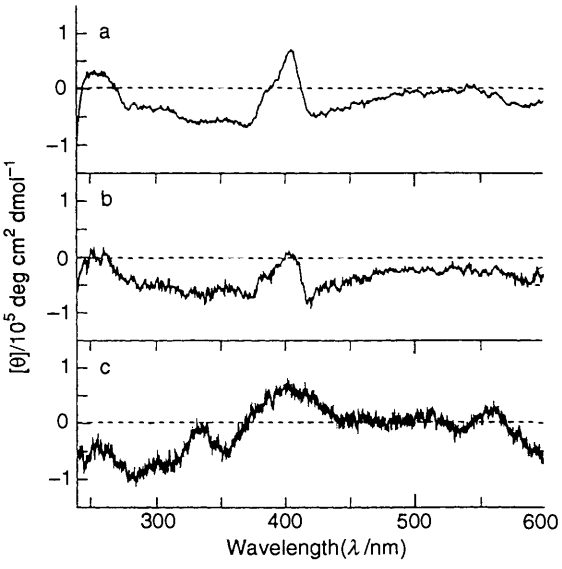


Fig. 1 CD spectra in the Soret band region. (a) Native cytochrome *c*; (b) PGL-Cytc; (c) PYR-Cytc. Concentration of each cytochrome *c* was 1.10×10^{-6} mol dm^{-3}.

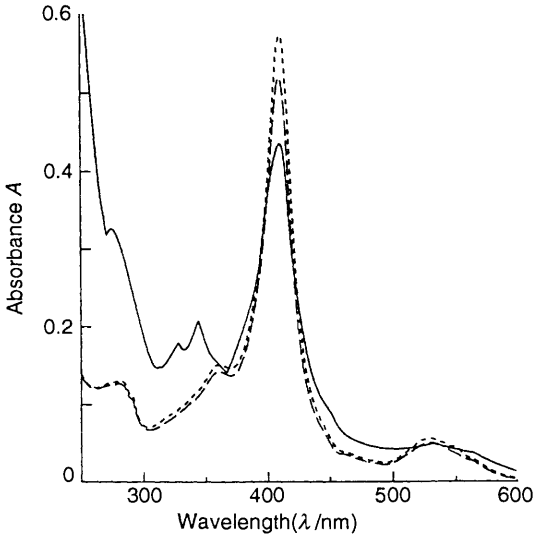


Fig. 2 Absorption spectra of mutant cytochromes *c*. (- - - -), Native cytochrome *c*; (– – –), PGL-Cytc; (——), PYR-Cytc. Concentration of each cytochrome *c* was 1.10×10^{-6} mol dm^{-3}.

rather than to the structural change around the haem region, since the Soret band peak has been reported not to shift for the largely disordered haem fragment I (1–65)[29] or even for urea-denatured cytochrome *c*.[42,43]

The two mutants showed a double-minimum-type CD spectrum with peaks at 210 nm and 222 nm, which may reflect α-helical structures in the protein (Fig. 3). Perturbation of the protein backbone structure was negligible with PGL-Cytc, supporting the idea that the replacement of Phe with pheGly did not cause a significant change in the structure of PGL-Cytc. However, the apparent α-helix content in PYR-Cytc was reduced to half that of native cytochrome *c*. The conformation of PYR-Cytc changed to some extent, forming a different conformation in which a pyrAla residue is located near the haem and interacts with aromatic side chains around the haem. This conformational change by substitution of the Phe with a bulky pyrAla should cause a polarity change inside the haem pocket by solvent relocation,[44] so that the redox properties might be affected.[23]

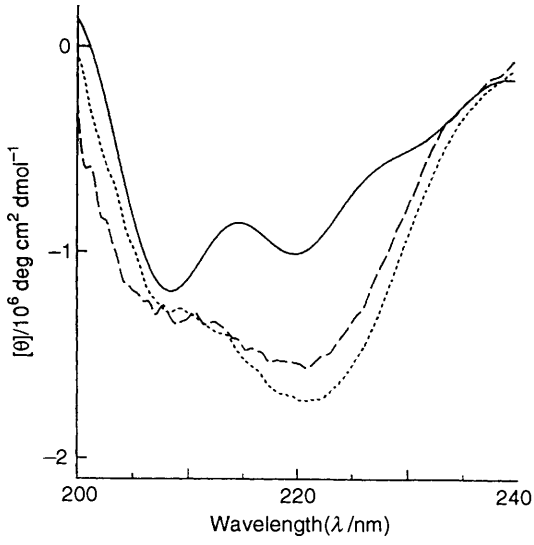


Fig. 3 CD spectra of mutant cytochromes *c* (200–240 nm region). (——), PYR-Cytc; (- - - -), native cytochrome *c*; (– – –), PGL-Cytc. Concentration of each cytochrome *c* was 1.10×10^{-6} mol dm^{-3}.

Reduction.—The haem moiety in a native ferricytochrome *c* was slowly reduced by 2-mercaptoethanol having an oxidation potential of ~0.30 V (Fig. 4a). Mutant cytochromes *c* in oxidized form were reduced by addition of 2-mercaptoethanol in 100 mmol dm^{-3} aq. sodium phosphate buffer (pH 6.9) at 25 °C. The kinetic constant was smaller in PGL-Cytc than in the native cytochrome *c*, indicating that the redox-potential gap between the haem moiety and the reductant is reduced by the substitution. On the other hand, iron(III) PYR-Cytc was reduced faster than was native cytochrome *c* (Fig. 4b). There are two possible mechanisms to explain the accelerated electron-transfer reaction observed in this experiment. First, the redox potential of the haem moiety decreased and an enhanced potential gap between iron(III) PYR-Cytc and the reductant (free-energy controlling). Second, substitution for the bulky pyrAla residue induced a structural change in the haem crevice, which probably facilitates access of reductant to the haem moiety (frequency-factor controlling). Spectroscopic analysis indicated a direct interaction between the haem and the pyrenyl group, suggesting that the free-energy term is controlling the electron transfer. However, since we have observed a conformational change in PYR-Cytc, the frequency factor may be another important factor determining the reduction rate in this case.

We examined the contribution of the frequency factor to the electron-transfer process of native cytochrome *c* or PYR-Cytc. Two kinds of reductants, butane-2-thiol and propane-1-thiol, were used for reduction of PYR-Cytc. The reaction rates with these bulky reductants were smaller than that with 2-mercaptoethanol either in reduction of native cytochrome *c* or in reduction of PYR-Cytc (Table 1.) Therefore, bulkier reductant molecules pass less easily through the haem crevice than does 2-mercaptoethanol, indicating that the haem moiety of PYR-Cytc is not exposed to the surface of the protein.

Next, we examined another two kinds of reductant 2-mercaptoethylamine and 2-mercaptoacetic acid. These reductants have a positive or negative charge, respectively, in a solution buffered at pH 6.9, while they are almost the same as 2-mercaptoethanol in molecular volume. In the case of native cytochrome *c*, the reduction rate with negatively charged 2-mercaptoacetic acid was very low. In contrast, positively charged 2-mercaptoethylamine reduced the cytochrome *c* at a rate comparable with that with 2-mercaptoethanol (Table 1). Native cytochrome *c* is a positively charged protein (pI = 11),

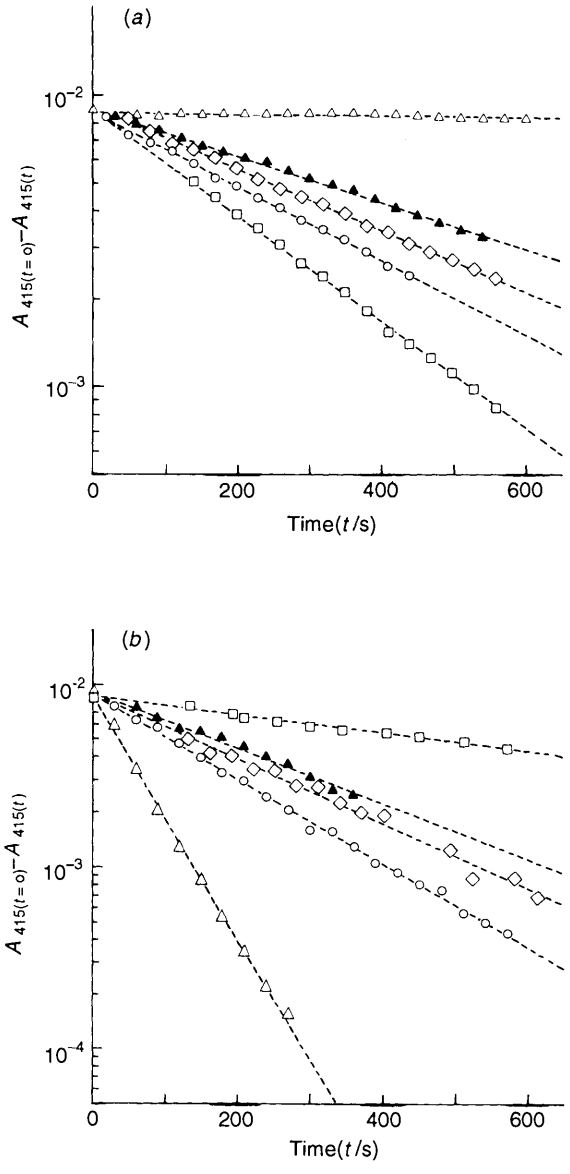


Fig. 4 Reduction kinetics of native cytochrome *c* and PYR-Cytc with various reductants. (a) Reduction kinetics of native cytochrome *c*; (b) Reduction kinetics of PYR-Cytc. ○, 2-mercaptoethanol; □, propane-1-thiol; ◇, butane-2-thiol; ▲, 2-mercaptoethylamine; △, 2-mercaptoacetic acid.

Table 1

Reductants	Reaction rate ($k/10^{-3}$ s^{-1})		
	PGL-Cytc	PYR-Cytc	Cytochrome *c*
$HSCH_2CH_2OH$	1.79	5.31	2.94
$HSCH_2CH_2Me$		1.16	4.18
$HSCH(Me)CH_2Me$		4.09	2.44
$HSCH_2CO_2H$		15.3	0.07
$HSCH_2CH_2NH_2$		3.51	1.84

which has lysine residues around the haem crevice,[45] but negatively charged aspartic acid or glutamic acid residues were located around or inside the haem crevice (Fig. 5). So, 2-mercaptoacetic acid has difficulty passing through the crevice probably because of repulsive electrostatic interactions.

In comparison, PYR-Cytc was reduced by either positively or negatively charged reductants. The passage found in PYR-Cytc was not affected by electrostatic interactions. This result

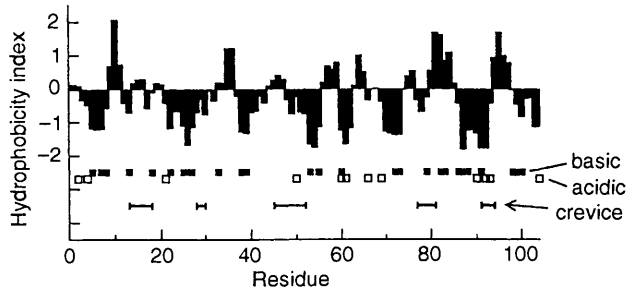


Fig. 5 Hydrophobicity profile of native cytochrome *c*. The amino acid positions are indicated on the *x*-axis; the hydrophobic (+) or hydrophilic (−) indices are shown on the *y*-axis. Bar chart, the hydrophobicity index of amino acid residues. ■, location of basic amino acid residue; □, location of an acidic amino acid residue; horizontal bar (|——|), the haem crevice is made up from the amino acids located in this region. The haem crevice has acidic and hydrophilic amino acid residues.

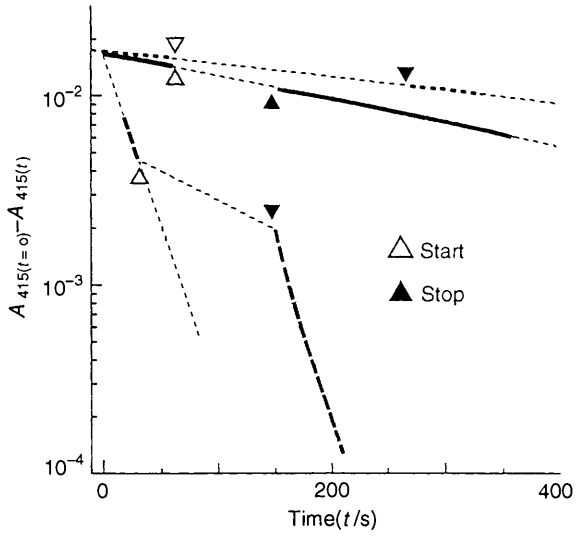


Fig. 6 Reduction kinetics of native and mutant cytochromes *c* with or without irradiation. (- - - -), PGL-Cytc; (——), native cytochrome *c*; (– – –), PYR-Cytc. Light irradiation was started at the time shown by open triangles (△), and was stopped at the time indicated by filled triangles (▼). During light irradiation, the reduction could proceed as represented by thin broken lines (- - - -).

indicates that substitution by the bulky pyrAla residue changed the polypeptide conformation and provided a novel passage of reductant in PYR-Cytc that was different from that in the native cytochrome *c*. This new passage might be either a new crevice through which reductants can access the haem or a new electron-transport pathway through the polypeptide matrix of PYR-Cytc provided by incorporation of the aromatic side chain of pyrenylalanine located at position 82 near the haem.

Effect of Photo-irradiation.—A pyrenyl residue is known to be a catalytic photosensitizer.[16] We examined the effect of photo-irradiation on the reduction reaction of PYR-Cytc. A reaction cuvette was irradiated by a UV light source (λ_{max} 330 nm) during reduction of the iron(III) mutant cytochromes *c* by 2-mercaptoethanol. Before and after irradiation, the concentration of iron(II) haem was monitored for 10 min (Fig. 6). Neither acceleration nor deceleration effects were observed for the native cytochrome *c* and PGL-Cytc. On the other hand, the reduction rate during the photo-irradiation decreased with PYR-Cytc. The duration of photo-irradiation was changed, and the reduction rate of PYR-Cytc was determined. After 100, 120 and 350 s of photo-irradiation, PYR-Cytc was reduced to

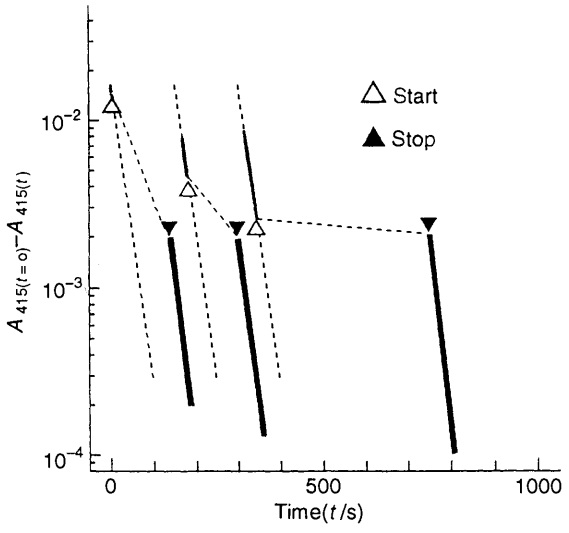
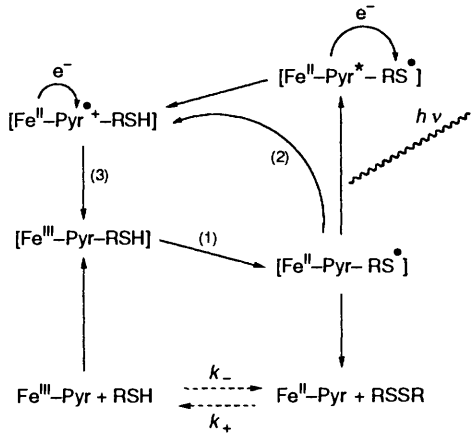


Fig. 7 Dependency of the reduction of PYR-Cytc on duration of light irradiation. (——), the absorbance change without light irradiation for 100, 120 and 350 s. The light irradiation was started at the time indicated by open triangles (△), and was stopped at the time indicated by filled triangles (▼). The thin broken lines (- - -) represent reduction reactions during light irradiation.



Scheme 2

almost the same degree of ~ 88% conversion (Fig. 7), indicating that the system reached equilibrium under photo-irradiation. Since the irradiation effect was not observed in the native cytochrome *c* and PGL-Cytc, excitation of the haem moiety is not the main reason for the observed deceleration of the reduction. Therefore, the haem should be oxidized through photosensitization by the pyrenyl group in PYR-Cytc. The potential gap of ~ 3 V between the ground state and the excited state of the pyrenyl residue[16] is sufficient to reduce an iron(III) haem moiety that has redox potential of 0.25 V. A possible reaction pathway is summarized in Scheme 2. First, a reductant (RSH) binds to PYR-Cytc (Pyr-Fe^{III}) and an electron is transferred from the reductant to haem (Step 1). When the pyrenyl residue is excited by light irradiation, an excited electron on the pyrenyl residue (Pyr*-Fe^{II}) is transferred to the radical RS$^{\bullet}$ (Step 2). This step generates a pyrenyl radical cation residue (Pyr$^{\bullet+}$-Fe^{II}) and RSH. Finally, an iron(III) haem is reformed through electron transfer from Fe^{II} to the pyrenyl radical cation (Step 3). The route passing through Steps 2 and 3 forms a reverse oxidation pathway of the haem during photo-irradiation. However, this Scheme remains to be tested by various spectroscopic methods.

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