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Cytochrome c_{552} from *Thermus thermophilus* Engineered for Facile Substitution of Prosthetic Group

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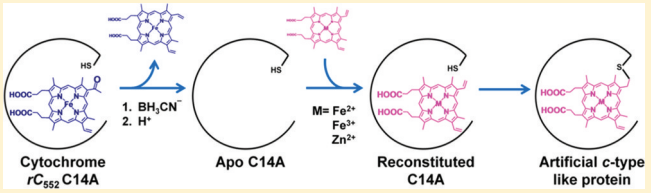
Supporting Information

ABSTRACT: The facile replacement of heme *c* in cytochromes *c* with non-natural prosthetic groups has been difficult to achieve due to two thioether linkages between cysteine residues and the heme. Fee et al. demonstrated that cytochrome c_{552} from *Thermus thermophilus*, overproduced in the cytosol of *E. coli*, has a covalent linkage cleavable by heat between the heme and Cys11, as well as possessing the thioether linkage with Cys14 [Fee, J. A. (2004) *Biochemistry* *43*, 12162−12176]. Prompted by this result, we prepared a C14A mutant, anticipating that the heme species in the mutant was bound to the polypeptide solely through the thermally cleavable linkage; therefore, the removal of the heme would be feasible after heating the protein. Contrary to this expectation, C14A immediately after purification (as-purified C14A) possessed no covalent linkage. An attempt to extract the heme using a conventional acid−butanone method was unsuccessful due to rapid linkage formation between the heme and polypeptide. Spectroscopic analyses suggested that the as-purified C14A possessed a heme *b* derivative where one of two peripheral vinyl groups had been replaced with a group containing a reactive carbonyl. A reaction of the as-purified C14A with $[BH_3CN]^-$ blocked the linkage formation on the carbonyl group, allowing a quantitative yield of heme-free apo-C14A. Reconstitution of apo-C14A was achieved with ferric and ferrous heme *b* and zinc protoporphyrin. All reconstituted C14As showed spontaneous covalent linkage formation. We propose that C14A is a potential source for the facile production of an artificial cytochrome *c*, containing a non-natural prosthetic group.



Cytochrome *c* (Cyt *c*) is one of the most ubiquitous proteins ranging from mammals to bacteria, with a primary function in the electron transport processes of both aerobic and anaerobic respiratory systems.[1,2] Properties of Cyt *c*, which include a small size, relatively high thermostability, water solubility, and a colored cofactor, facilitate the spectroscopic analyses of the protein using a wide variety of techniques. A great deal of 3D structural data from various sources illustrates the functional mechanisms of Cyt *c* at an atomic level. Thus, Cyt *c* has been used as a major resource for basic biochemical and biophysical studies on the folding and unfolding mechanisms of proteins,[3−6] intramolecular electron transfer mechanisms,[7,8] and specific interprotein interaction mechanisms.[9,10] In addition to these fundamental studies, the application of Cyt *c* in biomaterials research has emerged as a current topic. For example, oxidase activity introduced to Cyt *c* has been studied in the construction of artificial heme enzymes.[6,11−13] Methods for the modification of an electrode with Cyt *c* by various methods has also been developed to apply the protein to bioelectronic devices such as biomemory devices,[14−17] biodiodes,[18] and biofuel cells[19] and for use in bio-photoswitching functions.[8,20,21] The thioether bonds located between the polypeptide and the heme moiety in Cyt *c* contribute to improve stability of the biomaterials produced.[22] However, further development and evolution of the Cyt *c*-based materials are limited due to the stable covalent linkages and the difficulty in replacing the heme *c* with an artificial prosthetic group having non-natural function(s).[22,23] In order to overcome the limitations, engineered Cyt *c* should (i) contain a readily removable heme species, (ii) be capable of incorporating non-natural prosthetic groups through reconstitution, and (iii) have the ability to form covalent linkage(s) with the reconstituted external prosthetic group.

Ferguson and co-workers demonstrated that apo-Cyt c_{552} (Cyt c_{552} without heme *c*) from a thermophile, *Hydrogenobacter thermophilus* produced by Ag^+ treatment was able to incorporate ferrous heme *b* and form thioether bonds spontaneously, thereby enabling the full recovery of the original Cyt c_{552}.[24−27] They also succeeded in reconstitution of apo-Cyt c_{552} with other metalloprotoporphyrins utilizing zinc, manganese, and cobalt as the metal ions as well as employing a metal-free protoporphyrin.[26,28] These findings indicate that

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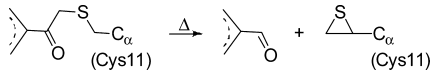
engineered Cyt *c* sourced from a thermophile potentially satisfies the above criteria (ii) and (iii) and may be useful in the objective of producing an engineered Cyt *c* possessing an artificial prosthetic group. However, in order to satisfy the criteria (i) complicated procedures required to prepare apo-Cyt c_{552} by a conventional method remain to be solved.

Fee and co-workers reported unusual heme-binding behavior in Cyt c_{552} from *Thermus thermophilus* (abbreviated to rC_{552} in their reports) where the protein was expressed and accumulated in the cytoplasm of *E. coli*.[29,30] In rC_{552}, a normal thioether bond was found between Cys14 and the heme-4-vinyl group of heme *b*, while another cystein residue, Cys11 formed an unprecedented bridge, "heme−CO−CH_2−S−CH_2−C_α(Cys11)", with the other vinyl group of the heme. The bridge was unstable and was cleavable by heat at higher pH than 6 affording formyl and thiirane groups (Scheme 1).[29]

Scheme 1. An Unprecedented Bridge in rC_{552} and Products after Its Cleavage Proposed by Fee et al.



Because of the remaining thioether bond, the heme derivative was irremovable from rC_{552} even after the heat cleavage of the unusual bridge.

These findings prompted us to prepare a Cys14 mutant of rC_{552}. We expect that the Cys14 mutant will possess a heme moiety that is solely conjugated to the thermally cleavable linkage with Cys11, and the heme would be removable from the polypeptide by heating the protein, with a subsequent extraction operation.

In this study, we prepared a C14A mutant of rC_{552} (C14A) where Cys14 is replaced with an alanine residue. Contrary to expectation, C14A contained a heme species that did not contain a covalent linkage with Cys11 immediately after the purification (as-purified C14A) despite that the protein was treated under pH 6. The conventional acid−butanone method was found to be inefficient at extracting the heme species, as the initial acidification step in the heme extraction process promoted a linkage formation between the heme species and Cys11. Spectroscopic analyses suggest that the heme species in as-purified C14A was a heme *b* derivative where one of the two vinyl groups had been modified to form a carbonyl group. A reaction of as-purified C14A with a hydride donor, $[BH_3CN]^-$, blocked the linkage formation, with subsequent acid−butanone extraction affording C14A in a heme-free apo-form (apo-C14A) in high yields. The quantitative reconstitution of apo-C14A with both ferric and ferrous heme *b* was followed by a spontaneous covalent linkage formation between the heme and polypeptide, probably through interactions between one of the vinyl groups of the heme and Cys11. Apo-C14A was found to accept facile incorporation of zinc protoporphyrin with subsequent covalent linkage formation enabling tethering to the Zn porphyrin in the polypeptide.

EXPERIMENTAL PROCEDURES

Chemicals. All chemicals were purchased from Nakarai Tesque, Wako Co., and Sigmal-Aldrich and used without further purification.

Expression and Purification of rC_{552} C14A Mutant. Gene of rC_{552} was amplified from chromosome of *Thermus thermophilus* HB8 by a PCR method using a sense primer, 5′-CATATGCAGGCGGACGGGGCCAAGATCTAC-3′, and an antisense primer, 5′-GGATCCTTACTTCAGGCC-GAGCTTCTTCTTCCG-3′, in which the *Nde*I and *BamH*I sites (underlined) were introduced to each primer, respectively. The sense primer was designed to skip a sequence corresponding to a signal peptide and attach a start codon (ATG) deriving from the *Nde*I site to the structural gene of cytochrome c_{552}. The amplified gene was inserted to pET-22b(+) expression vector (Novagen Co.) to give pET-rC_{552}. The mutant C14A was constructed by Quick Change Site-Directed Mutagenesis Kit (Stratagene Co.) using pET-rC_{552} as a temperate. The following sequence and its complementary were used as mutation primers, 5′-CCAGTGCGCGGGGGCT-CACCAGCAAAACG-3′. The underlined sequence corresponds to Ala of mutagenesis. A bacterial strain, *E. coli* BL21(DE3), was used as a host for overproduction of the rC_{552} C14A mutant.

A culture medium (150 mL LB added 50 μg/mL ampicilline) was inoculated from a plate of BL21(DE3) cells containing pET-rC_{552}. After incubation for 18 h at 30 °C, the culture was used to inoculate 7 L of a LB medium. This culture was incubated for 18 h with shaking at 37 °C in a jar fermenter at 180 rpm. No IPTG was added for induction. The cells were harvested by centrifugation at 7000*g* for 10 min and stored at −80 °C until use.

Purification of rC_{552} was performed according to the following protocol. The whole purification process was carried out at 4 °C unless specified. Frozen *E. coli* pellets (15 g) were thawed in ice and resuspended in 60 mL of 30 mM succinic acid−NaOH buffer, pH 5.0 (succinic buffer), along with DNase (for nucleotide removal) and sonicated. The suspension was then centrifuged at 17 500 rpm for 45 min, and the resultant supernatant was loaded into a pre-equilibrated CM-Sepharose column (120 mL) with succinic buffer and eluted with a linear gradient of 1 M NaCl of the same buffer. Reddish color fractions were collected and concentrated by ultrafiltration to 1 mL along with the buffer exchange to succinic buffer. The concentrated solution was passed through a Q-Sepharose column (5 mL) pre-equilibrated with succinic buffer to remove residual nucleotides and impurities. When further purification was required, an additional CM-Sepharose column (100 mL) pre-equilibrated with succinic buffer was applied after the Q-Sepharose column operation.

Concentration of purified proteins was determined by a Bradford method using cytoskeleton (Cytoskeleton Inc.) for protein staining.

Preparation of the rC_{552} C14A Mutant in Apo-Form (Apo-C14A). To the as-purified C14A protein was added 10 equiv of $Na[BH_3CN]$ at 25 °C in the succinic buffer, immediately followed by the acidification of the solution (pH 1.8) and subsequent butanone extraction. The noncovalently bound heme (heme *b*) moved into the organic phase, with the aqueous phase containing the apoprotein subjected to dialysis against the succinic buffer. The resultant aqueous solution was added $(NH_4)_2SO_4$ to achieve a final concentration of 2 M and then loaded on a pre-equilibrated butyl-Sepharose column (70 mL) with the succinic acid buffer containing 2 M $(NH_4)_2SO_4$ (pH 5.0). The apo-C14A was eluted by a linear gradient against the succinic acid buffer. Fractions containing apo-C14A were identified by values higher than 7.0 in the ratio of absorbance at 280 over 420 nm and were used for further investigations.

Reconstitution of Apo-C14A with Heme *b* and Zn-PPIX. Reconstitution of apo-C14A was achieved by mixing

equimolar amounts of ferric or ferrous heme *b* with apo-C14A at 25 °C in the succinic buffer. Hemin dissolved in alkaline water (5 mM, pH 10.0) was mixed with 10 μM apo-C14A in the succinic buffer. The initial product (a cytochrome *b* species) was typically obtained in 30 min. For the subsequent covalent linkage formation between the polypeptide and the incorporated heme *b* to occur, the reaction mixture was left under ambient conditions. The reaction was discontinued when no change was observed in the visible spectrum of the reaction mixture. Under the present reaction conditions, it took for 9 h to complete the reaction. For the reconstitution with ferrous heme *b*, an excess amount of dithionite salt was added to both the alkaline hemin solution and the apo-C14A succinic buffer solution prior to the mixing. All operations were performed in a globe box filled with Ar gas.

Reconstitution with Zn-PPIX was performed by mixing apo-C14A with Zn-PPIX in a 1:1 ratio. An aqueous solution of Zn-PPIX (5 mM) was prepared by dissolution in alkaline water (pH 10.0), followed by addition to the succinic buffer containing 10 μM apo-C14A at 25 °C under dark conditions. Following the incorporation of Zn-PPIX onto apo-C14A (typically finished in 30 min), a covalent linkage between the polypeptide and the incorporated Zn-PPIX was formed spontaneously. Under the present reaction conditions, it took for 48 h to complete the reaction.

Spectroscopy. UV−vis spectra were recorded on a MultiSpec-1500 spectrometer equipped with a temperature controller (Shimadzu Co.). Circular dichroism spectra were recorded on a J-720WN CD spectrometer with a temperature controller (JASCO Co.). A thin optical cell (1 mm path length) containing 10 μM protein solution in succinic buffer was used for measurements. When determining a melting point, temperature was raised linearly from 20 to 95 °C at a rate of 1 °C min^{-1}. All data were corrected for at 222 nm, which corresponds to an absorption maximum of a negative Cotton effect by α-helices.

Mass spectra of proteins and the extracted heme were recorded on ultraflexIII TOF/TOF (MALDI-TOF mass spectrometer, Bruker Daltonics Co.) and JMS-700 (FAB mass spectrometer, JEOL Co.), respectively. α-Cyano-hydroxy-*trans*-cinnamic acid and Magic Bullet were used as the matrix of the MALDI-TOF and FAB mass measurements, respectively. The MALDI-TOF instrument was calibrated with myoglobin (*m*/*z* values, 16 952) from horse heart prior to measurements. All protein samples were subjected to a dialysis against 5 mM ammonium acetate (pH 5.0) before the measurements.

IR spectra were recorded on a FT/IR 6100 spectrometer (JASCO Co.) equipped with an ATR unit. Samples were mounted on the reflection window of the ATR unit without dilution by any salts or oils.

Resonance Raman spectra were obtained on a SpectraPro-300i spectrometer (Acton Research) with a 2400-groove grating, a holographic Supernotch filter (Kaiser Optical Systems), and a LN-1100PB CCD detector (Princeton Instruments) cooled with liquid N_2. The excitation light at 413 nm was obtained from a Kr^+ laser (Spectra-Physics). Spectra were collected with a spinning frozen sample positioned in a backscattering geometry. The sample was sealed in a NMR tube and kept in liquid N_2 in a double-walled, low-temperature quartz Dewar vessel. Power at the sample was less than 5 mW. Peak frequencies were calibrated relative to toluene and CCl_4 standards (accurate to ±1 cm^{-1}).

RESULTS

The C14A Mutant Contained an Unprecedented Heme Species. Similar to the overproduction of the original rC_{552},[31] *E. coli* cells contain the expression plasmid for the rC_{552} C14A mutant (C14A), appearing deep red in color when cultured in a LB medium. The overproduced C14A was successfully purified in a holo-form from the cytosol of *E. coli* using ordinary column chromatography. A MALDI-TOF mass spectrum of as-purified C14A showed two major peaks at *m*/*z* = 14 273.9 and 14 905.8 (Figure 1A). The former peak

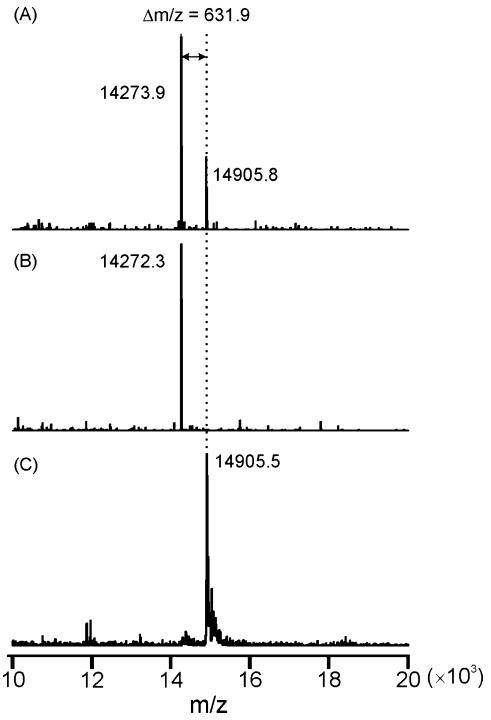


Figure 1. Mass analysis of as-purified C14A. A peak attributable to holo-C14A (*m*/*z* = 14 905.8) converges on another peak attributable to apo-C14A (14 273.9) as laser power rises. Panels A and B were recorded at lower laser power (20% of the maximum) and higher laser power (60% of the maximum) of the instrument. (C) C14A after acidification recorded at the laser power of 20% of the maximum.

agrees with the calculated molecular mass of C14A without an attached prosthetic group (apo-C14A, M_w = 14 271.7), with the latter peak assigned to apo-C14A + 632. The latter peak diminished as the laser power of the instrument rose, eventually converging on the former peak (Figure 1B). This suggests that a heme species in the as-purified C14A does not have a covalent linkage with the polypeptide of C14A unlike heme *c*. Ellman's reagent test showed that more than 80% of the as-purified C14A possessed a thiol group assignable to Cys11 in C14A. Therefore, Cys11 retains its thiol group in as-purified C14A, in almost quantitative amounts, without forming a covalent bond. Although the mass analysis suggests the absence of a covalent linkage between the polypeptide and heme species in as-purified C14A, a conventional acid−butanone method[32] was inefficient at extracting the heme species, resulting in the quantitative recovery of a heme binding protein. Heme staining of SDS-PAGE gel by the resultant protein showed that the heme species was associated with the protein by covalent linkage(s) (Figure S1). Further experiments revealed that acidification of the protein solution (pH ∼ 2) accelerated the

linkage formation in the as-purified C14A, which accounted for the inefficiency of the acid−butanone extraction. After acid treatment, Ellman's reagent test revealed the absence of a free thiol group, indicating that Cys11 participated in the linkage formation. Acid-treated C14A showed a major peak at 14 905.5 in MALDI-TOF mass spectrum, which is identical to that of as-purified C14A in the holo-form (14 905.8) (Figure 1C). Under increased laser power conditions, there was no peak observed, which corresponded to the C14A apo-form. These results indicate that no apparent change in the composition of C14A accompanies the linkage formation. Key features of the UV−vis spectra of the as-purified and acid-treated C14A mutants are listed in Table S1.

Preparation of the Apo-C14A Mutant. The mass of the heme species calculated from the difference in the masses of as-purified holo- and apo-C14As ($\Delta m/z$ = 632, Figure 1) has been assignable to heme *b* (M_w = 616.2) with a single oxygen atom attached. Using this assignment, we assumed that the heme species in the as-purified C14A is a heme *b* derivative where one of the two vinyl substituents has been replaced with a group containing a carbonyl group. Along this assumption, the observed linkage formation may be attributed to nucleophilic attack by a cysteinyl thiol on the putative carbonyl group with the assistance of a proton. On the basis of this theory, we attempted to block the reaction of the carbonyl group with the cysteinyl thiol via a reaction of the as-purified C14A with a hydride, $[BH_3CN]^-$, prior to the acid−butanone extraction. The reaction was effective in preventing the covalent linkage formation both in the ferric and ferrous forms, allowing the removal of more than 90% of the heme species to yield apo-C14A. UV−vis spectra of as-purified C14A in the ferrous form showed a blue shift by 2 nm upon the reaction with the hydride (Figure 2). The resultant heme species did not exhibit a band at

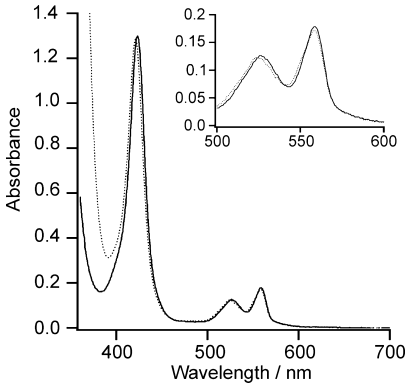


Figure 2. UV−vis spectra of as-purified C14A in the reduced form before (solid line) and after (dotted line) the reaction with $Na[BH_3CN]$.

572 nm, confirming that the removable heme species formed in as-purified C14A is different from that formed in the original rC_{552} (the heme species in p572).[29] Further purification was achieved by a hydrophobic column, which was effective in separating the residual holo-protein possessing the covalently attached heme species. The UV−vis spectrum showed up to 98% purity of the resultant apo-C14A, after the final column separation (Figure S2).

The heme species extracted from the organic phase was identified as heme *b* using FAB-mass (m/z = 616) and IR spectra (Figure S3). Other than heme *b*, no apparent vestige of a heme species was found in the spectra. It is proposed that heme *b* is obtained from the heme *b* derivative via the hydride reduction of the carbonyl group to alcohol and subsequent OH^- elimination with the assistance of a proton to form the vinyl group. We will discuss this point later in the spectroscopic characterization of the heme species.

The CD spectrum of apo-C14A at 20 °C showed the negative Cotton effect assignable to α-helices, which occurred to the same extent as those observed for holo-C14A and the Cyt c_{552} wild type (Figure S4A). This observation infers that apo-C14A is resistant to denaturation and retains a conformation similar to as-purified holo-C14A and the wild type. SEC for apo-C14A consistently showed a major elution volume virtually identical to that of holo-C14A and the wild type (Figure S4B), demonstrating that apo-C14A has a comparable volume to those of holo-C14A and the wild type. Therefore, the apo-C14A retains a compact conformation without denaturation, in spite of loss of the heme species.

A melting point of apo-C14A (T_m, calculated from temperature dependence of the CD data at 222 nm) was not obtained in the normal buffer solution (20 mM MES-NaOH, pH 6.0) up to 95 °C but was observed at 48.6 °C in the presence of 2 M guanidine·HCl (Figure S5A). Among the *c*-type cytochromes reported so far, Cyt c_{552} from *Hydrogenobacter thermophilus* (*Ht* Cyt c_{552}) has shown the highest thermostability in the apo-form.[27,33] The T_m value for *Ht* Cyt c_{552} in the apo-form (apo-Ht Cyt c_{552}) is reported to be 51 °C (phosphate buffer, pH 7.3) in the absence of guanidine·HCl, emphasizing the high thermostability of apo-C14A although the comparison of the T_m values is only speculative due to the different measurement conditions.

Reconstitution of Apo-C14A with Heme *b*. Previous studies have found that apo-Cyt *c* sourced from *H. thermophilus* or horse heart requires a reducing agent to cleave the intramolecular disulfide bond of the two cysteine groups prior to reconstitution with heme *b*; otherwise, the disulfide bond blocks the incorporation of the heme to a cavity in the apoproteins.[24,25] This may be true of apo-C14A if the intermolecular disulfide bond between two Cys11 molecules is formed, as this bond would cause the two apo-C14A molecules to have their cavities facing close to each other, preventing access by the heme. However, apo-C14A retained the thiol group in more than 80% of Cys11 even under aerobic conditions. Accordingly, reconstitution of apo-C14A in the present study could be performed without any prior reduction reactions. Removal of dioxygen from the reaction mixtures was performed only when ferrous heme *b* was used for the reconstitution.

The titration of apo-C14A to ferric heme *b* (10 μM) at 25 °C and pH 5.0 is shown in Figure 3A. Bands characteristic of ferric heme *b* was replaced with those assignable to a six-coordinated ferric cytochrome (determined from the λ_{max} of the soret band; see Table 1).[33] No further change was observed after titration of a stoichiometric amount of apo-C14A to heme *b*. Titration of apo-C14A to ferrous heme *b* also accompanied the emergence of a typical spectrum of six-coordinated ferrous cytochromes (Figure 3B and Table 1). Similar to the case of ferric heme *b*, saturation of the spectral change was observed when an equimolar amount of apo-C14A was added. These results indicate that apo-C14A is able to quantitatively incorporate both ferric and ferrous heme *b*. Immediately after the reconstitution, the resultant ferric and ferrous cytochromes were subjected to the pyridine hemochrome analysis.

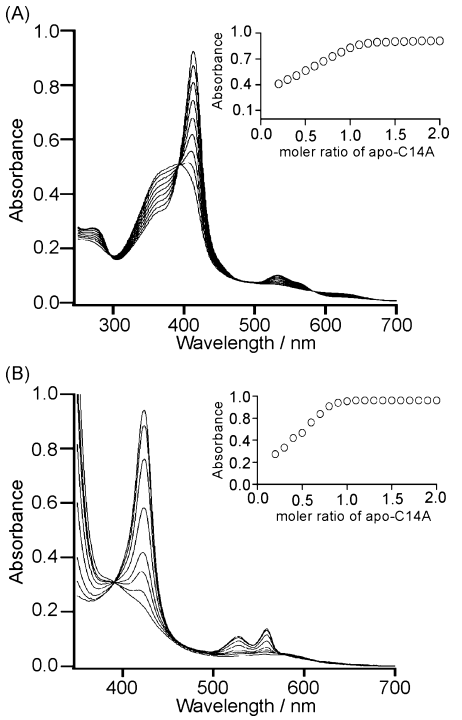


Figure 3. Spectrophotometric titration of apo-C14A to (A) ferric and (B) ferrous heme *b*. The apoprotein is reconstituted with heme *b* to yield cytochrome *b*. The insets display profiles of the titrations monitored at the emerging soret bands of the corresponding cytochromes *b*.

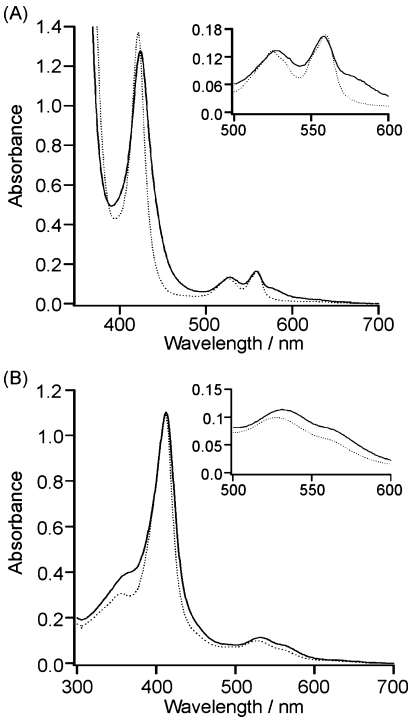


Figure 4. UV−vis spectra of the C14A mutant reconstituted with (A) ferrous and (B) ferric heme *b*. The spectra immediate after the reconstitution (solid lines) show progressive blue shifts (dotted lines) under ambient conditions.

Table 1. Absorption Maxima of C14A Reconstituted with Ferric and Ferrous Heme *b* Recorded Immediately after the Reconstitution and after the Covalent Linkage Formation

	λ_{max}/nm (ε/cm^{-1} mM^{-1})	
	ferric heme *b*	ferrous heme *b*
immediately after the reconstitution	413 (99)	424 (128)
	532 (11), 564 (8)	528 (13), 558 (17)
after forming the covalent linkage	411 (98)	421 (137)
	527 (9)	524 (13), 559 (17)

The spectra showed an α-band at 556 nm for both the reconstituted ferric and ferrous heme *b*, indicating that the incorporated heme *b* has two vinyl substituents.[24] Therefore, cytochrome *b* is an immediate product of the reconstitution.

After reconstitution, ferrous cytochrome *b* showed a progressive blue shift in its UV−vis spectrum (Figure 4A and Table 1). A similar observation was reported when a thioether bond was formed between the heme and polypeptide in apo-*Ht* Cyt c_{552} reconstituted with ferrous heme *b*.[34] In accordance with the report, the heme staining on SDS-PAGE gels for the resultant protein revealed a heme covalently bound to the polypeptide (Figure S6). The pyridine hemochrome spectrum showed the α-band at 553.0 nm, which is characteristic of a heme *b* derivative where one of the two vinyl groups is saturated.[24] A thiol group in the resultant protein was not detected using Ellman's reagent. These results indicate that ferrous cytochrome *b* can form a covalent linkage between one of the two vinyl groups of the heme and thiol group of Cys11. Ferric cytochrome *b* also showed a spontaneous blue shift in the UV−vis spectrum (Figure 4B and Table 1). The heme staining (Figure S6), pyridine hemochrome spectrum (the α-band, 553.0 nm), and Ellman's reagent test supported formation of the same covalent linkage in ferric cytochrome *b* to that observed in ferrous cytochrome *b*. This result is distinctive of the ferric cytochrome *b* obtained from apo-C14A. When apo-Cyts *c* from other sources are reconstituted with ferric heme *b*, the ferric cytochrome *b* products either do not form a covalent linkage or form different linkages from those observed in the corresponding ferrous cytochrome *b*[24,25,35] where the mechanism responsible for the different reactivities has not been elucidated. The results presented here are opposite to previous reports and may provide a clue to the different reactivities of previously studied ferric and ferrous cytochromes *b* in the formation of the covalent linkage.

Reconstituted C14A showed higher thermostability than apo-C14A (Figure S5B). The T_m values of reconstituted C14A in the ferric form were determined to be 57.6 and 57.9 °C before and after the covalent linkage formation, respectively, in the presence of 2 M guanidine·HCl. The results indicate that the incorporation of the heme improves the thermostability of C14A, although the T_m of the ferric Cyt c_{552} wild type is much higher (73.0 °C under the same measurement conditions; Nakajima, unpublished data).

Redox potentials were determinded to be 374 ± 5 mV (vs SHE) for the reconstituted apo-C14A. No change was observed before and after the covalent linkage formation. The result revealed a significant positive shift of the redox potential from that of the wild type (232 mV).[36] It is unlikely that the shift is asscociated with the difference in the peripheral substituents (i.e., the vinyl or thioether group) since the covalent linkage formation that is accompanied by the conversion of the vinyl group to a thioether group showed no effect on the redox potential. A slight divergence of coordination sturctures of

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heme sepcies in reconstituted C14A and the wild type may cause the shift.

Reconstitution of Apo-C14A with Zinc Protoporphyrin(IX). Changes in the UV−vis spectra occurring with titration of apo-C14A to 10 μM of zinc protoporphyrin-(IX) (Zn-PPIX) at pH 5.0 are shown in Figure 5. A spectrum

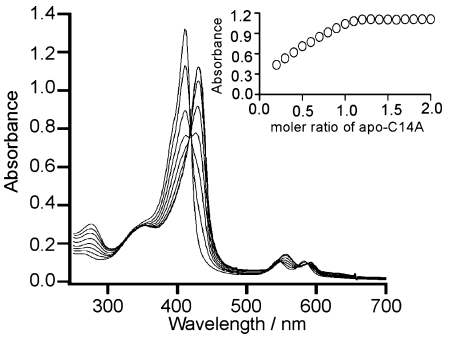

Figure 5. Spectrophotometric titration of apo-C14A to Zn-PPIX. The apoprotein is reconstituted with Zn-PPIX to yield a corresponding Zn-substituted cytochrome. The inset displays a profile of the titration monitored at the emerging soret band of the cytochrome.

consistent with Zn-PPIX was replaced during the titration to a spectra corresponding to a holo-protein bearing the non-covalently attached Zn-PPIX (λ_{max} = 430, 555, and 592 nm).[28] A pyridine metallochrome spectrum of the reaction mixture showed the α-band identical to that of free Zn-PPIX at 587 nm, indicating that the two vinyl substituents of Zn-PPIX remain intact in the protein at this stage. Similar to the titration to ferric and ferrous heme *b* performed previously, the spectral change reached saturation when a stoichiometric amount of apo-C14A was added. A progressive blue shift in a visible spectrum was also observed for reconstituted C14A with Zn-PPIX (Zn-C14A) (Figure 6). The spectrum observed after the

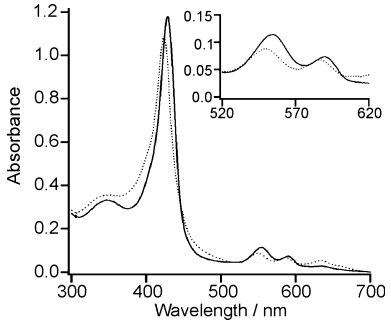

Figure 6. Spectral change of C14A reconstituted with Zn-PPIX. The UV−vis spectrum immediately after the reconstitution (solid line) shows spontaneous blue shift (dotted line).

shift showed bands at 424, 551, and 586 nm. A similar observation was described in a previous study on the reconstitution of *Ht* apo-Cyt c_{552} with Zn-PPIX, and this was used as an index of the thioether bond formation between the peripheral vinyl groups and cysteinyl thiols in the polypeptide.[28] Acid−butanone extraction consistently resulted in the detection of trace amounts of Zn-PPIX in the organic phase, with a colored species remaining in the aqueous phase (Figure S7). This supports the finding that the heme is covalently attached to the polypeptide. The pyridine metallochrome spectrum of Zn-C14A showed the α-band at 585 nm, which is located at the midpoint of the spectrum for Zn-PPIX (587 nm) and for zinc-substituted wild type Cyt c_{552} (580 nm), indicating that one of the vinyl groups of Zn-PPIX has formed a covalent linkage in Zn-C14A. Ellman's reagent test performed after the covalent linkage formation did not detect a thiol present in the polypeptide. Since a trace amount of the C14A dimer was found throughout the experiment, it is plausible that Cys11 participated in the covalent linkage with the vinyl group.

The temperature dependence of the CD spectra in 2 M guanidine·HCl (Figure S5C) provided T_m values of 58.5 and 63.5 °C before and after the covalent linkage formation, respectively. The latter value is rather close to that of the zinc-substituted wild type Cyt c_{552} (69.1 °C in the presence of 2 M guanidine·HCl; Nakajima, unpublished data). The covalent linkage formation seemed to improve the thermostability of Zn-C14A comparable to that of zinc-substituted Cyt c_{552} prepared by a conventional method.

Spectroscopic Characterization of the Heme Species in As-Purified C14A. The present study arrived at three findings regarding the heme species in the as-purified C14A: (1) the molecular mass of the heme species was calculated to be 632, which is assignable to heme *b* (616) + 16, (2) the heme species is transformed to heme *b* through reduction by a hydride species and subsequent reaction with a proton, and (3) the heme species forms a covalent linkage with the cysteinyl thiol with the assistance of the proton. These findings are consistently explained by postulating that the heme species in the as-purified C14A is a heme *b* derivative in which one of the vinyl groups is replaced with a group containing a carbonyl group. In order to obtain experimental evidence to support to the postulation, resonance Raman (rR) spectroscopy for C14A was performed.

The spectrum of ferric C14A before and after the covalent linkage formation by the acid treatment is shown in Figure 7. The normal-mode frequencies assigned in the present spectra[37] are summarized in Table 2. At the lower frequency region (Figure 7B), bands assignable to $\delta(C_\beta C_A C_B)$ and $\delta(C_\beta C_C C_D)$ (see atoms labeling in Figure 8) were observed at 412 and 382 cm^{-1}, respectively.[29,38−40] As expected, the heme species is shown to have the same peripheral groups as heme *b*. These bands appear virtually identical in intensity and frequency before and after the covalent linkage formation, indicating that the vinyl and propionic substituents do not participate in the reaction. In addition, virtually no change was observed in the ν_3, ν_4, and ν_{10} bands after the linkage formation, revealing that the reaction did not accompany any alterations in the electronic and coordination states of the heme species (Figure 7A). Contrastingly, apparent changes were observed for the ν_2 and ν_{11} bands. Since these bands are assignable to $\nu(C_\beta C_\beta)$ and are mixed with the vibrational modes of periphery substituents,[40] the observed changes suggest that a peripheral group of the heme species, excluding the propionate and vinyl groups, participated in the linkage formation.[29]

A clue to which peripheral group participated in the linkage formation was obtained from the alteration of bands observed around 1630 cm^{-1}. Upon formation of the covalent linkage, the band present at 1627 cm^{-1} diminished, with a weak band emerging at ~1630 cm^{-1}, observed as a shoulder of ν_{10} band. Although the mode frequency of C=C stretching from vinyl groups of heme *b* show a band in this region,[37] it is unlikely to assign the diminished band to that of $\nu(C_A C_B)$, since the band of $\delta(C_\beta C_A C_B)$ (412 cm^{-1}) remains unchanged before and after

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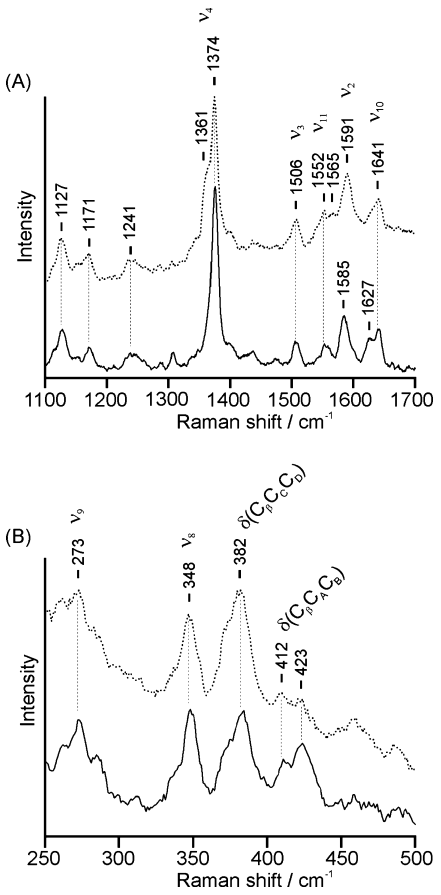


Figure 7. (A) High-frequency and (B) low-frequency resonance Raman spectra of C14A in the ferric form. Solid line, as-purified C14A (before the covalent linkage formation); dotted line, acid-treated C14A (after the covalent linkage formation). Spectral traces are offset for the purpose of illustration. The excitation wavelength was 413.1 nm.

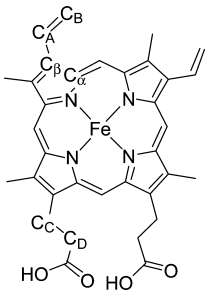


Figure 8. Atom labeling of heme *b* used in Table 2.

the covalent linkage formation (Figure 7B). Therefore, we tentatively assign the band at 1627 cm^{-1} to ν(C=O) of a peripheral carbonyl group. Using this assignment, the diminishment of the band is attributed to a reaction of the carbonyl group accompanying the linkage formation. The band at ~1630 cm^{-1} is then assignable to $\nu(C_AC_B)$, which was possibly obscured in ν(C=O) band before the linkage formation and unveiled by the diminishment of ν(C=O) band. Similar to the behavior observed around 1630 cm^{-1}, a band at 423 cm^{-1} showed a significant decrease in intensity after the linkage formation. This may also be related to the disappearance of the carbonyl group; however, assignment of the band at 423 cm^{-1} has not been confirmed due to a lack of precedent data.

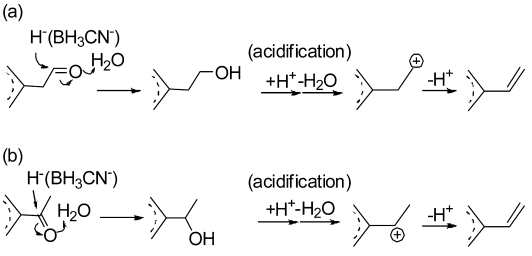
Table 2. Resonance Raman, Normal-Mode Frequencies (cm^{-1}) of C14A before and after the Covalent Linkage Formation

normal mode	as-purified	covalent linkage formed
ν_{10}	1641	1641
ν_2	1585	1591
ν_{11}	1552	1552, 1565
ν_3	1506	1506
ν_4	1376	1376
δ ($C_\beta C_A C_B$) (vinyl)	412	412
δ ($C_\beta C_C C_D$) (propionate)	383	382
ν_8	348	348
ν_9	273	273

DISCUSSION

Mutagenesis at Cys14 Brought Unexpected Properties to rC_{552}. Previous reports by Fee et al.[29] demonstrated that when Cyt c_{552} from *T. thermophilus* was produced in the cytoplasm of *E. coli*, the protein (rC_{552}) contained an unusual bridge between Cys11 and the heme-2-vinyl group (Scheme 1), where the normal thioether bond between Cys14 and the heme-4-vinyl group remained. On the basis of their findings, we prepared a Cys14 mutant of rC_{552} and attemtped to yield an apo-form of the C14A mutant. However, results obtained from experiments employing the Cys14 mutant (C14A) were contrary to the expected scenario. Although the C14A did not possess a covalent linkage between the heme species and polypeptide immediately after purification (as-purified C14A), a conventional acid–butanone extraction did not work due to covalent linkage formation between the heme species and polypeptide during the extraction process. Results obtained from investigations into the linkage formation confirm that the heme species in the as-purified C14A is a heme *b* derivative in which one of the vinyl groups is replaced with a group containing a carbonyl group. There are two possible structures of the carbonyl group formed from the vinyl group, i.e., acetyl and aldehyde groups, which are indistinguishable in the rR data conducted for the present study. However, since heme *b* is the major product of the hydride reduction and protonation reaction, this suggests the acetyl group as the likely candidate for the carbonyl group in the heme *b* derivative (Scheme 2).

Scheme 2. Formation of the Vinyl Group from Possible Carbonyl Groups in the Heme *b* Derivative: (a) Aldehyde and (b) Acetyl Groups

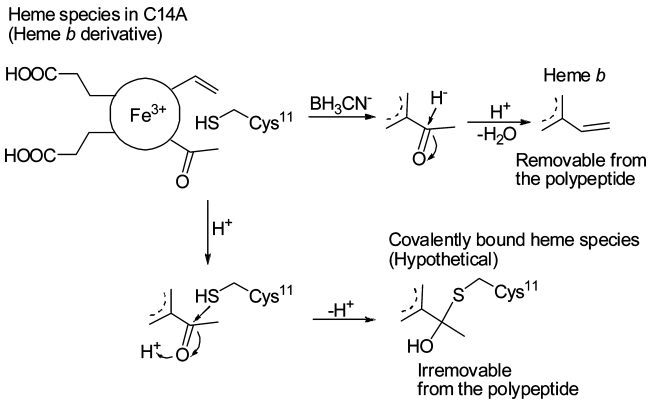


Under the present reaction conditions (pH ~ 2), the aldehyde group produces a primary alcohol that is generally inactive for the following OH^- elimination (Scheme 2a). Therefore, a heme *b* derivative having an alcohol group should be found after the aldehyde reaction, which is, inconsistent with the present result where recovery of heme *b* predominantly occurred. In contrast, a secondary alcohol would be the product of the reaction with

the acetyl group (Scheme 2b), which forms a carbocation stabilized by the π-conjugation system of the porphyrin ring, facilitating the smooth production of the vinyl group.

The proposed reactions of the as-purified C14A are summarized in Scheme 3. Unlike original rC_{552}, the heme *b* derivative in the as-purified C14A is modified to the acetyl group at one of the vinyl groups and has no linkage with Cys11, which accounts for the behavior of the reactions observed in the present study (Scheme 3). The identification of the covalently bound heme species is now in progress to enable the conclusive confirmation of the proposed scheme. The final products described in Scheme 3 could also be feasibly obtained by postulating an epoxide rather than the acetyl group. However, the rR data is negative for the involvement of the epoxide in the heme species. An epoxide ring would show a band corresponding to its breathing mode at around 1250 cm^{-1},[41] and the heme species bearing the epoxide would exhibit some spectral changes upon the covalent linkage formation. However, such apparent changes attributed to the epoxide were not observed in the corresponding region of the present rR spectra (Figure 7A).

Scheme 3. Proposed Mechanisms of the Reactions Observed in the Present Study



Reconstitution of Apo-C14A. Reaction of the as-purified C14A with $[BH_3CN]^-$ prior to an acid–butanone extraction converted the heme species in the as-purified C14A to heme *b*, which allowed the production of apo-C14A in high yields. Apo-C14A was then successfully reconstituted with stoichiometric amount of ferric and ferrous heme *b* to afford cytochrome *b*, which subsequently underwent spontaneous transformation to new cytochromes, with each bearing a single covalent linkage between Cys11 and one of the heme vinyl groups in heme *b*. The resultant cytochromes from ferric and ferrous cytochrome *b* were indistinguishable in the pyridine hemochrome spectra, suggesting that the covalent linkage reactions were identical in ferric and ferrous cytochromes *b*. The reconstitution of *Ht* Cyt c_{552} in an apo-form with ferrous heme *b* was followed by the covalent linkage formation between the polypeptide and heme *b* to recover original Cyt c_{552}, while different products were obtained when reconstitution occurred with ferric heme *b*.[24] It is proposed that since *Ht* Cyt c_{552} in the apo-form has a partially deformed structure, reconstitution with the ferric and ferrous heme *b* results in different conformations, and only a protein binding ferrous heme *b* is suitable to subsequently form the correct thioether bonds.[28,35] This is likely a consequence of the small difference in the positioning of ferric and ferrous heme *b* in the protein cavity potentially affecting the reactivity of the heme vinyl group to the cysteinyl thiol. The present study reveals that apo-C14A retains a rigid and compact conformation comparable to holo-C14A and the Cyt c_{552} wild type. This infers that apo-C14A retains a vacant cavity that is ready to embed heme *b* regardless of its oxidation state. Consequently, ferric cytochrome *b* possibly also has a conformation amenable to form the covalent linkage observed for ferrous cytochrome *b*.

In order to examine the versatility of apo-C14A as a scaffold for artificial cytochromes, we attempted the reconstitution of apo-C14A with zinc protoporphyrin (Zn-PPIX) because of wide uses of zinc-substituted Cyt *c* in basic and applied studies.[8,42,43] Similar to the findings for heme *b*, Zn-PPIX was found to be readily incorporated into apo-C14A. Full reconstitution to the holo-form was achieved by the stoichiometric addition of Zn-PPIX to an apoprotein, which demonstrated the tight binding of Zn-PPIX to apo-C14A. These findings reveal that binding to the cavity of apo-C14A is not specific to heme *b* and has the potential to accommodate a metalloprotoporphyrin possessing a different ion.

Immediately after reconstitution, the pyridine metallochrome spectrum of holo-C14A revealed that the two vinyl groups of Zn-PPIX remained intact and a covalent linkage was not formed upon incorporation into the protein cavity. The initial product of the reconstitution subsequently formed a covalent linkage between Zn-PPIX and the polypeptide. Similar to the case of ferric and ferrous heme *b*, we attributed one of the vinyl groups and Cys11 to this linkage based on the results of Ellman's reagent test and the pyridine metallochrome analysis. According to the T_m values, apo-C14A reconstituted with Zn-PPIX exhibits high thermostability. After covalent linkage formation, the thermostability approached that of Zn-substituted wild type Cyt c_{552}, which is a distinctive property of the mutant, revealed by the use of this artificial protein. The results obtained here propose Zn-C14A as a candidate for future studies investigating the properties of zinc-substituted cytochrome *c*.

CONCLUSION

Replacement of the Cys14 of rC_{552} with alanine resulted in the production of an unexpected heme protein, which contained the intact Cys11 and a heme *b* derivative possessing an acetyl group attached to its peripheral. Using this mutant (C14A), we succeeded in exchanging the original prosthetic group with a metalloprotoporphyrin and achieved subsequent covalent linkage formation between the polypeptide and metalloprotoporphyrin. These achievements satisfy the criteria of a desirable engineered Cyt *c* as described earlier in this paper. The C14A mutant showed high thermostability when derived from the original Cyt c_{552} in holo- and apo-form and from all reconstituted proteins. The rigid and compact conformation of the apo-form seems to account for the high ability of C14A to form the correct covalent linkage with heme *b*, irrespective of its oxidation state. Thus, the present study concludes that C14A is a potential resource of thermostable artificial cytochromes *c*, which is prepared by the facile exchange of the original prosthetic group with an artificial species. Studies on the reconstitution of C14A with organometallics other than metalloprotoporphyrins are now in progress to extend the versatility of C14A in the creation of artificial organometallic proteins based on Cyt c_{552}.

ASSOCIATED CONTENT

Supporting Information

Figures S1–S8 and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

SEC, size exclusion chromatography; CD, circular dichroism; MES, 2-morpholinoethanesulfonic acid; CV, cyclic voltammogram; SHE, standard hydrogen electrode.

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