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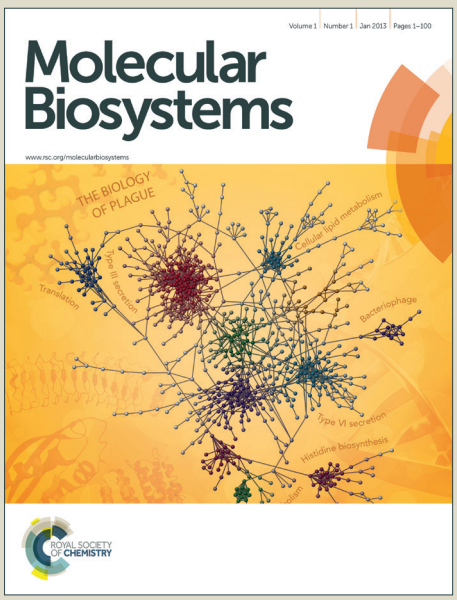





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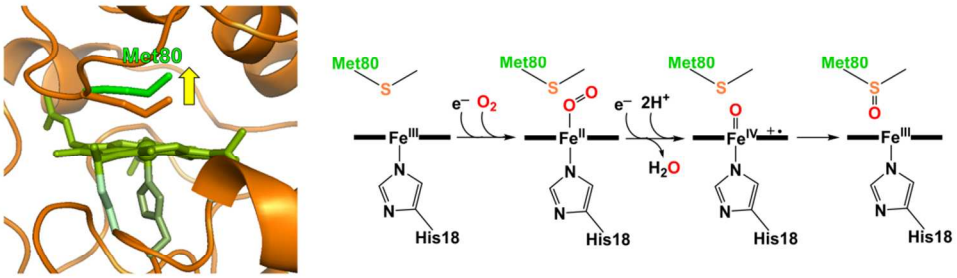
**Graphical Abstract**

Met80 of cyt *c* is oxidized site-specifically by reaction of the heme with molecular oxygen in the presence of a reducing agent, when Met80 dissociates from the heme iron due to interaction of the protein with cardiolipin.



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ARTICLE

# Self-oxidation of Cytochrome *c* at Methionine80 with Molecular Oxygen Induced by Cleavage of the Met−Heme Iron Bond†

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Zhonghua Wang,[ab#] Yuki Ando,[a#] Ari Dwi Nugraheni,[a] Chunguang Ren,[a] Satoshi Nagao[a] and Shun Hirota*[a]

Met80 of cytochrome *c* (cyt *c*) has been shown to dissociate from its heme iron when cyt *c* interacts with cardiolipin (CL), which triggers the release of cyt *c* to the cytosol initiating apoptosis. We found that the mass of human cyt *c* increases 16 Da at the Met80–Lys86 region by reaction with molecular oxygen in the presence of CL-containing liposomes and dithiothreitol (DTT). To investigate the effect of Met80 dissociation on the reaction of cyt *c* with molecular oxygen without affecting its secondary structures, a human cyt *c* mutant (Δ8384 cyt *c*) was constructed by removing two amino acids (Val83 and Gly84) from the loop containing Met80. According to MALDI-TOF-MS and tandem mass measurements, Met80 of Δ8384 cyt *c* was modified site-specifically to methionine sulfoxide when purified in the presence of molecular oxygen, whereas Met80 was not modified in the absence of molecular oxygen. A red-shift of the Soret band from 406 to 412 nm and absorption increase at ~536 and ~568 nm were observed for Δ8384 cyt *c* when it reacted with DTT and molecular oxygen, followed by a further red-shift of the Soret band to 416 nm and absorption increase at ~620 and ~650 nm. These results indicate that Met80 of cyt *c* is oxidized site-specifically by formation of the oxy and subsequent Compound I-like species when Met80 dissociates from the heme iron, where the Met80 modification may affect its peroxidase activity related to apoptosis.

## Introduction

Cytochrome *c* (cyt *c*) is a heme-containing protein, which transfers electrons from cytochrome $bc_1$ complex to cytochrome *c* oxidase. Cytochrome *c* oxidase uses the electrons and protons to reduce molecular oxygen to water at the inner membrane of mitochondria, whereas cyt *c* exists in the intermembrane space.[1,2] Cyt *c* also plays a key role in apoptosis, where it is released to the cytosol when permeabilization of the mitochondrial outer membrane occurs.[3,4] Cyt *c* contains three long α-helices surrounding its heme prosthetic group. The heme is covalently attached to the polypeptide through two thioether linkages, and His18 and Met80 are axially ligated to the heme iron.[5-7] Met80 dissociates relatively easily from the heme iron, resulting in an opening of the heme crevice.[8-14] For example, Met80 has been reported to dissociate from the heme iron under alkaline conditions,[8-11] in the presence of various denaturants,[12,13] and at high temperatures.[14]

Interaction of cyt *c* with cardiolipin (CL) in mitochondria plays an important role in mediating the release of cyt *c* to cytosol and initiating apoptosis.[15-18] Cyt *c* exhibits a less packed tertiary conformation upon interaction with CL.[19] Cyt *c* interacts with CL electrostatically and hydrophobically.[20] The electrostatic interaction of cyt *c* with negatively charged CL-containing membranes unfolds the C-terminal α-helical region of cyt *c*, assisting formation of further hydrophobic interactions between cyt *c* and CL.[21] It is proposed that the hydrophobic interactions are involved by inserting an acyl chain of CL into the hydrophobic core of cyt *c*.[22] Met80 of cyt *c* dissociates from the heme iron when cyt *c* interacts with CL.[23] The redox potential of cyt *c* shifts negatively by 350–400 mV upon interaction with CL, and the peroxidase activity of cyt *c* increases dramatically.[24] It has been reported that the cyt *c*-CL complex oxidizes CL by the peroxidase reaction of cyt *c*.[25,26]

[a]*Graduate School of Materials Science, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan. E-mail: hirota@ms.naist.jp; Fax: (81) 743-72-6119; Tel.: +81-743-72-6110*

[b]*Chemical Synthesis and Pollution Control Key Laboratory, College of Chemistry and Chemical Engineering, China West Normal University, Nanchong 637002, China.*

† Electronic Supplementary Information (ESI) available: Experimental details, MALDI-TOF mass spectra and Tandem mass spectrum. See DOI: 10.1039/b000000x/

# These authors contributed equally to this work.

The C-terminal region of horse cyt *c* has been shown to domain swap between molecules through its hinge loop (Thr78−Ala83), associated with perturbation of its Met80−heme iron bond.[27] The peroxidase activity of cyt *c* has also been shown to increase by the domain-swapped dimerization, owing to the dissociation of Met80 from the heme iron.[28] A mutant iso-1-cyt *c* (mutation of trimethyllysine 72 to alanine) has also been shown to increase the peroxidase activity compared to its native protein by dissociation of Met80 from the heme iron.[29] It has also been reported that the peroxidase activity of carboxymethylated cyt *c* is higher than that of native cyt *c*, due to the disruption of the Met80–heme bond.[30]

Proteins suffer structural changes and functional disorders by oxidation of its amino acid. They are oxidized when oxidative stress arises from a variety of situations, such as the aging process and lifestyle-related disease.[31] Although all the residues in the proteins are susceptible to oxidation by a reactive oxygen species, the methionine residue is particularly sensitive to oxidation and is converted to methionine sulfoxide. It has been reported that methionine oxidation of cyt *c* in lens cells is related to cataracts,[32] and methionine-*S*-sulfoxide reductase A (MsrA) may repair the methionine sulfoxide to native methionine.[32,33] The two methionine residues of horse cyt *c* have been shown to be oxidized by reaction with various strong oxidants, such as hydroxyl radical (·OH),[34] singlet oxygen ($^1O_2$),[35] and hypochlorous acid (HOCl).[36] In this study, we show that Met80 of reduced cyt *c* is selectively oxidized with a mild oxidant, molecular oxygen, when Met80 dissociates from the heme iron. A possible reaction mechanism for the Met80 oxidation is proposed.

## Results and discussion

### Oxidation of cyt *c* with molecular oxygen by interaction with CL

A peak was observed at m/z = 12,235 in the MALDI-TOF mass spectrum of ferric wild-type human cyt *c* (Fig. 1, curve a). The mass value corresponded well within experimental error to that of positively charged wild-type human cyt *c* (m/z 12,234). After incubation of ferric wild-type human cyt *c* (12 μM) with DTT (500 μM, a mild reducing agent of the heme) and liposomes (consisted of 120 μM DOPC and 120 μM CL, DOPC:CL = 1:1) under air, an additional peak was observed at m/z = 12,251 in the mass spectrum (Fig. 1, curve b). The mass of the new species was 16 Da larger than that of wild-type human cyt *c*, indicating oxidation of cyt *c* by incorporation of a single oxygen atom during the incubation. However, no additional peak was observed at about m/z = 12,251 in the mass spectrum of wild-type human cyt *c* after incubation of the protein with DTT and DOPC liposomes (not containing CL) under air (Fig. 1, curve c). The peak at m/z = 12,251 was also not observed when wild-type human cyt *c* was incubated with DTT and DOPC/CL liposomes (DOPC:CL = 1:1) under $N_2$ atmosphere instead of air (Fig. 1, curve d). These results indicate that human cyt *c* was oxidized by reaction with molecular oxygen in the presence of a reducing agent and liposomes containing CL.

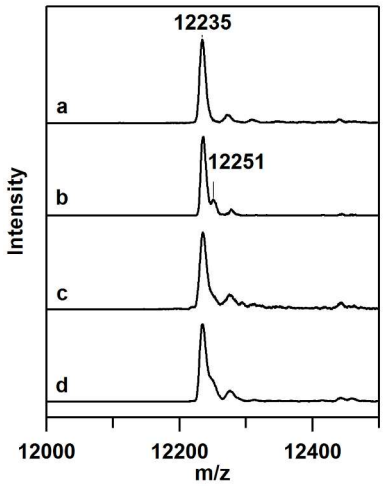


**Fig. 1** MALDI-TOF mass spectra of ferric wild-type human cyt *c* (a) before and (b,c,d) after incubation with liposomes in the presence of DTT. The protein was incubated with (b,d) DOPC/CL (1:1) and (c) DOPC liposomes under (b,c) air or (d) $N_2$ atmosphere. Incubation conditions: cyt *c* concentration, 12 μM; DOPC concentration, 120 μM; CL concentration, 0 or 120 μM; DTT concentration, 500 μM; buffer, 25 mM HEPES buffer; pH, 7.4; incubation time, 3 h; room temperature.

Wild-type human cyt *c* was digested with lysyl endopeptidase after incubation with DTT and liposomes, and the obtained peptide fragments were subjected to ESI-MS analysis to identify the position of oxidation (Fig. 2). In the mass spectrum of peptide fragments obtained by digestion of wild-type human cyt *c*, a peak was observed at m/z = 807.48, which corresponded to the mass of the protonated MIFVGIK peptide (calculated monoisotopic mass (Mm): 807.48) (Fig. 2, curve a). Additional peaks were observed at m/z = 786.60 and 823.48 in the mass spectrum of the peptide fragments obtained by digestion of cyt *c* after incubation in the presence of DTT and CL-containing liposomes. We attributed the peak at m/z = 786.60 to DOPC, since the Mm of protonated DOPC is 786.60 Da and the peak was detected in the spectrum of the fragments obtained by digestion after incubation with DTT and DOPC liposomes without CL. The mass of 823.48 Da was 16 Da larger than that of the MIFVGIK peptide (Met80–Lys86), showing that an amino acid in the MIFVGIK region containing Met80 was oxidized.

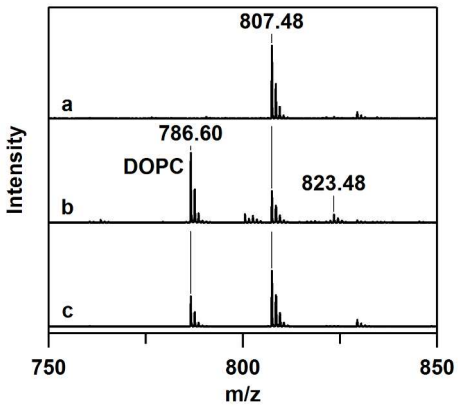


**Fig. 2** ESI-MS spectra of the peptide fragments obtained by digestion of wild-type human cyt *c* (a) before and (b,c) after interaction with liposomes in the presence of DTT. The protein was incubated with (b) DOPC/CL (1:1) and (c) DOPC liposomes under air, and subsequently digested with lysyl endopeptidase. Incubation conditions were the same as those in Fig. 1.

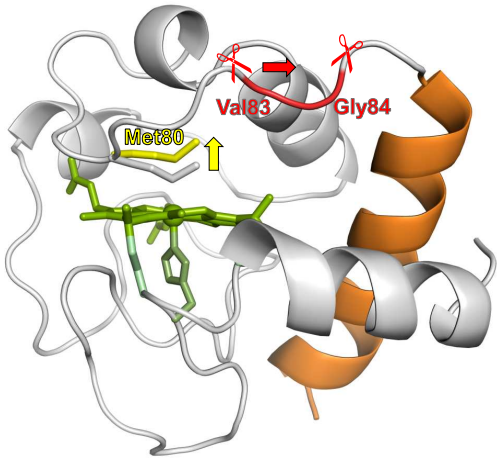


**Fig. 3** Schematic view of structural perturbation of cyt *c* by deletion of Val83 and Gly84. His 18, Met80, and the heme are shown as stick models. Met80 is shown in yellow, Val83 and Gly84 in red, C terminal α-helix in orange, and His18 and heme in green. The arrows represent the expected movement of the protein main chain and Met80 side chain by deletion of Val83 and Gly84. The coordinates of horse cyt *c* (PDB: 1HRC) are used.

### Characterization and oxidation of Δ8384 cyt *c*

Met80 of ferric cyt *c* has been shown to dissociate from the heme iron when cyt *c* interacts with CL.[23] We assumed that the above-mentioned oxidation of cyt *c* was induced by the dissociation of Met80 from the heme iron. However, wild-type human cyt *c* is easily degraded by incubation with CL-containing liposomes in the presence of DTT under air, which made it difficult to further study the wild-type protein. We have shown previously that cyt *c* molecules exchange the C-terminal α-helix between molecules in the domain-swapped dimer and trimer.[27] The C-terminal α-helix of the dimeric and trimeric cyt *c* interacted with the protein moiety in a similar manner as in the monomer, whereas the position of the hinge loop (Thr78–Ala83) was altered by the oligomerization. We envisaged that when we delete some amino acids from the loop at Pro71–Ile85 of cyt *c* (Ω loop before the C-terminal α-helix), the C-terminal helix will still interact with the protein moiety in a similar manner as in the wild-type protein but the rest of the loop will move toward the C-terminal α-helix, and thus Met80 will dissociate from the heme iron (Fig. 3). Therefore, to investigate the effect of Met80 dissociation from the heme iron on protein modification, we constructed Δ8384 human cyt *c*, in which Val83 and Gly84 in the Ω loop containing Met80 were deleted and the Met80−heme iron bond perturbed.

The Soret band of ferric Δ8384 cyt *c* blue-shifted to 406 nm from that of ferric wild-type cyt *c* at 410 nm (Fig. 4A). In addition, the Met S−$Fe^{III}$ charge transfer band at 695 nm[12,14,37] observed in the absorption spectrum of ferric wild-type cyt *c* was not observable in that of ferric Δ8384 cyt *c* purified with the $N_2$-bubbled buffer (Fig. 4A). These results indicated that Met80 dissociated from the heme iron of cyt *c*, due to deletion of the two amino acid residues (Val83 and Gly84) in the loop region. The CD spectrum of Δ8384 human cyt *c* (purified with a $N_2$-bubbled buffer; see text below) exhibited two negative bands at 208 and 222 nm, and was similar to that of wild-type cyt *c* (Fig. 4B). These results show that Δ8384 cyt *c* was constructed mostly of α-helices as the wild-type protein, and Met80 dissociated from the heme iron without significantly damaging the secondary structures.

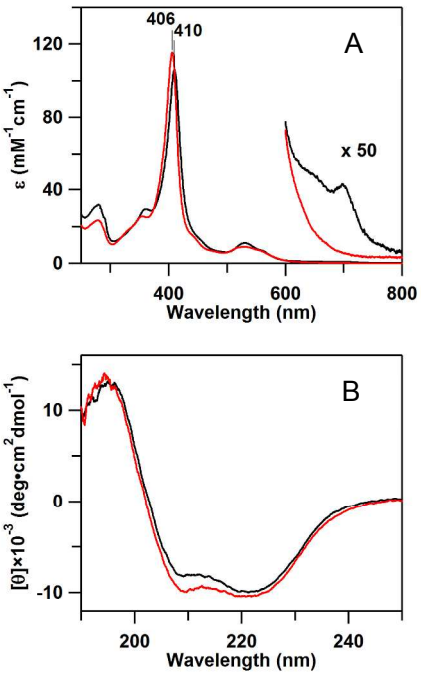


**Fig. 4** (A) Absorption spectra of ferric wild-type (black) and Met-unmodified (native) Δ8384 (red) human cyt *c*. Expansion of the absorption in the 600−800 nm region is also shown. Δ8384 human cyt *c* without modification of Met80 was purified from *E. coli* using $N_2$-bubbled buffer. The y-axis represents the extinction coefficient. Measurement conditions: cyt *c* concentration, 7 μM; buffer, 50 mM potassium phosphate buffer; pH, 7.0; temperature, 25 °C. (B) CD spectra of ferric wild-type (black) and Δ8384 (red) human cyt *c*. Each concentration of the protein was adjusted according to the intensity of its Soret band. Measurement conditions: cyt *c* concentration, 10 μM; buffer, 50 mM potassium phosphate buffer; pH, 7.0; room temperature.

The MALDI-TOF mass spectrum of ferric Δ8384 cyt *c* exhibited two peaks at m/z = 12,077 and 12,093 when it was overexpressed in *E. coli* and purified under air (Fig. 5A, curve a). The mass value of the peak at m/z = 12,077 corresponded well to that of positively charged Δ8384 cyt *c* (m/z 12,078), whereas the mass value of m/z = 12,093 corresponded well to that with an increase of 16 Da from the mass of Δ8384 cyt *c*. When the protein was purified using $N_2$-bubbled buffer, the peak at m/z = 12,093 was not observable, whereas the peak at m/z = 12,077 was observed (Fig. 5A, curve b). No further oxidation was observed in the MALDI-TOF mass spectrum of Δ8384 cyt *c* when its ferric form was exposed to air for several days. These results indicate that Met80 of Δ8384 cyt *c* is oxidized by the reaction with molecular oxygen through its ferrous form. Since only one additional peak was observed with a mass increase of 16 Da when Δ8384 cyt *c* was purified under air, a special amino acid residue in the protein may have been modified with an addition of an oxygen atom by reaction with molecular oxygen when the protein was purified under air.

In proteins, the methionine residue is sensitive to oxidation, resulting in formation of a methionine sulfoxide. The produced methionine sulfoxide can be reduced to methionine by MsrA in the presence of DTT.[32,38] To investigate the possibility of oxidation of a methionine residue in Δ8384 cyt *c*, the modified Δ8384 cyt *c* was treated with MsrA. The relative intensity of the mass peak at m/z = 12,093 compared to that at m/z = 12,077 of Δ8384 cyt *c* decreased significantly after incubation with MsrA and DTT, whereas it did not change significantly by incubation with only DTT (Fig. 5B). These results strongly suggest that an oxygen atom was attached to a methionine residue in Δ8384 cyt *c* by reaction with molecular oxygen.

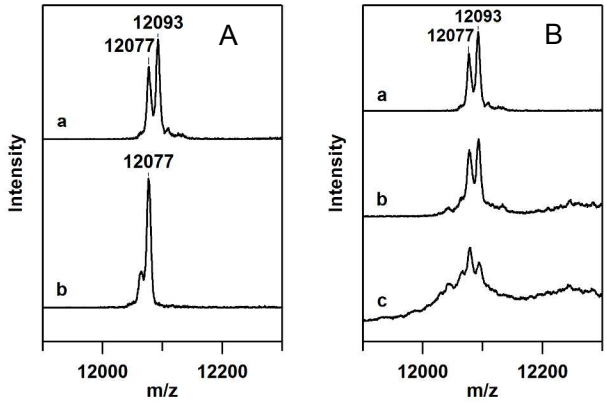


**Fig. 5** MALDI-TOF mass spectra of Δ8384 human cyt *c*. (A) Δ8384 human cyt *c* purified under air with (a) non-treated and (b) $N_2$-bubbled buffer. (B) Δ8384 human cyt *c* before and after treatment with MsrA: (a) as-purified Δ8384 cyt *c*, (b) treated with DTT, and (c) treated with MsrA and DTT. Δ8384 cyt *c* was purified under air. Reaction conditions: Δ8384 cyt *c* concentration, 70 μM; MsrA concentration, 0.2 mg/ml; DDT concentration, 20 mM; buffer, 50 mM potassium phosphate buffer, pH, 7.0; incubation temperature, 37 °C; incubation time, 2 h; under air.

**Identification of the modified residue of oxidized Δ8384 cyt *c***

We digested the Δ8384 cyt *c* protein purified under air, to elucidate the location of the modified methionine. The peptide fragments obtained by digestion with trypsin were analyzed with ESI-MS spectroscopy. All of the peptide fragments with masses higher than 500 Da were observed in the ESI-MS spectrum (Fig. 6): m/z = 547.26, 651.39, 678.38, 906.51, 1168.61, 1428.66, 1649.58, and 2007.96 (calculated Mm for protonated fragments: 547.27, 651.39, 678.38, 906.53, 1168.62, 1428.68, 1649.61, and 2007.97, respectively). However, an additional peak was observed at m/z = 667.38 in the ESI-MS spectrum. Three peptide fragments of trypsin-treated Δ8384 cyt *c* contain methionine. Two of the methionine-containing peptide fragments, IFIMK (amino acids 9–13) and MIFIK (amino acids 80–84), contain the same amino acid composition (I, I, F, M, K) and possess the same monoisotopic mass of 650.38 Da (Mm of its protonated ion: 651.39), although they have different amino acid sequences. The mass value of the additional peak in the ESI-MS spectrum corresponded well to that of an addition of 16 Da to the mass of the methionine-containing peptide fragment IFIMK or MIFIK, indicating that one of these fragments was modified. No additional peak was



 

observed at m/z = 2024, which corresponded to the mass increase of 16 Da from that of the peptide fragment GIIWGEDTLMEYLENPK (amino acids 56–72) containing the other methionine (m/z = 2007.96) (Fig. 6B). The peak at m/z = 667.38 was the only peak with a mass increase of 16 Da from that of a peptide fragment of Δ8384 cyt *c* obtained by the trypsin digestion.

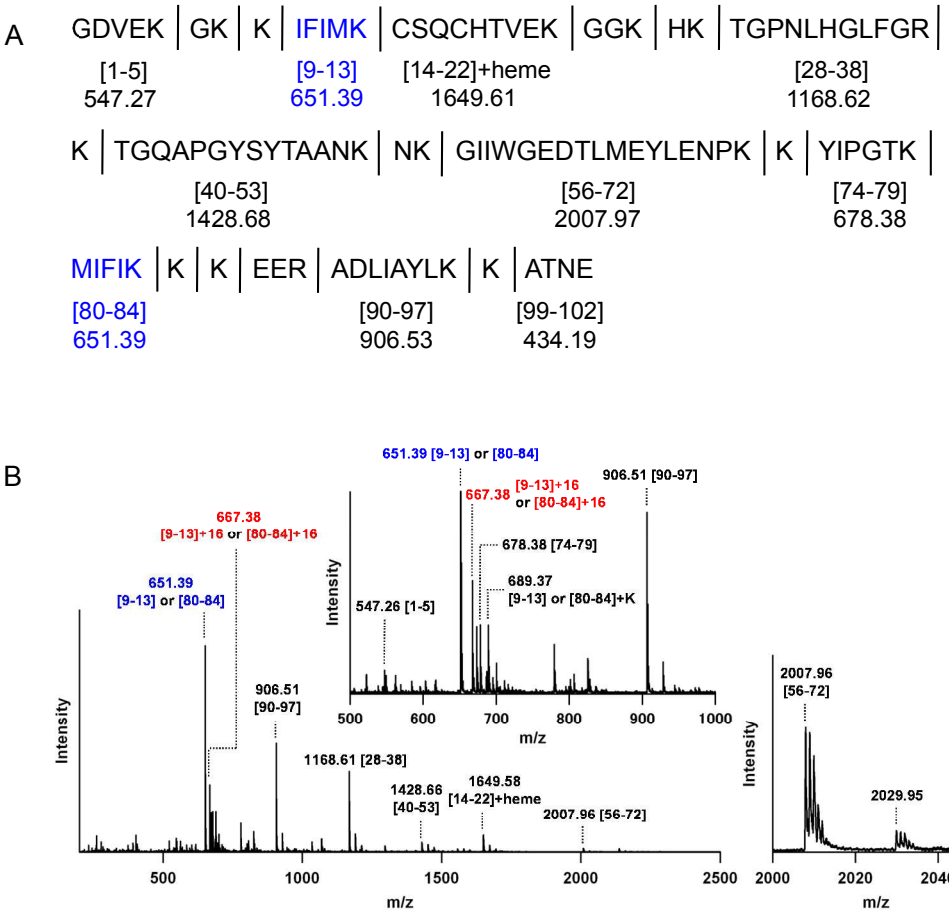


**Fig. 6** Peptide fragments and ESI-MS spectrum of trypsin-digested Δ8384 human cyt *c*. (A) Expected peptide fragments obtained by trypsin digestion of Δ8384 human cyt *c*. The number inside the bracket represents the amino acid sequence number of the peptide, and that under the bracket represents the monoisotopic mass of its protonated molecular ion. (B) ESI-MS spectrum of the peptide fragments of Δ8384 human cyt *c* purified under air. Expansions of the mass spectra in the 500−1,000 and 2,000−2,050 m/z regions are also shown.

High performance liquid chromatography (HPLC) analysis was performed for the fragments obtained by trypsin digestion of Δ8384 cyt *c* purified under air, and the observed fragment peaks were assigned by their masses defined by the MALDI-TOF mass measurements. Peaks for all the fragments consisting of four or more amino acids were observed in the HPLC chromatogram (Fig. 7A). In addition, the modified peptide fragment of m/z = 667.4 was observed as a single peak in the chromatogram. To identify the modified fragment (IFIMK or MIFIK) and modified residue, the tandem mass measurement was performed for the peptide fragment of m/z = 667.4. Mass peaks corresponding to $[IFIK]^+$ (m/z: 520.5, Mm: 520.4), $[FIK]^+$ (m/z: 407.3, Mm: 407.3), $[IK]^+$ (m/z: 260.3, Mm: 260.2), and $[K]^+$ (m/z: 147.2, Mm: 147.1) were all observed in the tandem mass spectrum of the peptide fragment of m/z = 667.4 (Fig. 7B), showing that the amino acid sequence of the modified peptide fragment has the sequence of IFIK. In addition, a peak was observed at m/z = 120.2, which was equivalent to the mass of an immonium ion of methionine (Mm: 104.1) with an increase of 16 Da.[39] These results show that the methionine of the MIFIK fragment (amino acids 80–84) was modified, i.e. Met80 of Δ8384 cyt *c* was oxidized to methionine sulfoxide.

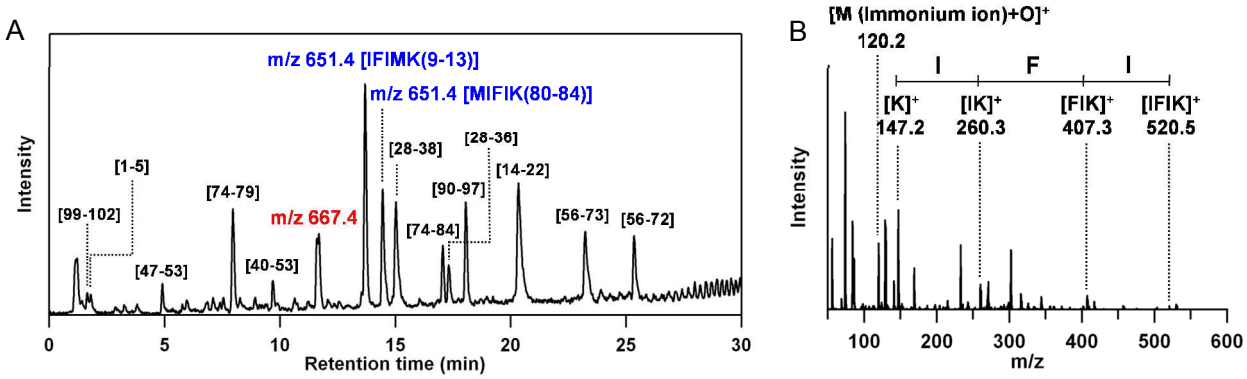


**Fig. 7** (A) HPLC chromatogram of the peptide fragments obtained by trypsin digestion of Δ8384 human cyt *c* purified under air. (B) Tandem mass spectrum of the peptide fragment with the mass of m/z = 667.4

**Mechanism of Met80 oxidation**

The reaction of Met80-unmodified ferric Δ8384 cyt *c* (purified with $N_2$-bubbled buffer, 12 μM) with molecular oxygen was investigated by monitoring the changes in its absorption spectrum in the presence of DTT (500 μM) at 25 °C in 50 mM potassium phosphate buffer, pH 7.0, under air. After addition of 500 μM DTT under air to Δ8384 cyt *c*, the Soret band red-shifted from 406 to 412 nm with a decrease in intensity and generation of two new absorption bands at ~536 and ~568 nm (Figs. 8A and 8B). These spectral characters of Δ8384 cyt *c* were similar to those of the oxygen complexes of the M80A cyt *c* mutant (537 and 570 nm),[40] oxymyoglobin,[41] and Compound III (oxy form) of horseradish peroxidase (HRP).[42] The wavelengths of the two new absorption bands at ~536 and ~568 nm were also similar to those of the oxy complex (~540 and ~570 nm) of dimeric cyt *c* obtained by reaction with a high concentration of hydrogen peroxide.[28] Therefore, an oxy complex of Δ8384 cyt *c* may have formed by the reaction with molecular oxygen in the presence of DTT. It has been reported that the Met80−heme iron bond of cyt *c* is destabilized or disrupted by interaction with CL,[23,43] and diatomic molecules such as CO and NO can bind to the cyt *c*:CL complex.[43,44] The cyt *c*:CL complex in the reduced state may have the ability to bind molecular oxygen and modify Met80 when Met80 dissociates from the heme iron. However, the absorption bands at ~536 and ~568 nm were not observed when DTT was added to Δ8384 cyt *c* under $N_2$ atmosphere, supporting the hypothesis that Δ8384 cyt *c* was oxidized by the reaction with molecular oxygen through its ferrous form.

Further incubation of ferric Δ8384 cyt *c* with 500 μM DTT under air caused a gradual red shift of the Soret band from 412 to 416 nm. Simultaneously, the absorption band at ~536 nm decreased slightly in intensity and a broad absorption band appeared at 600−700 nm in the absorption spectra (Figs. 8B and 8C). The difference spectra of each absorption spectrum with the initial absorption spectrum after the addition of DTT exhibited two absorption bands at ~620 and ~650 nm (Fig. 8C). These spectral features resemble those of Compound I (ferryl-oxo porphyrin π cation radical, [$Fe^{IV}$=O $Por^{+}$]$^{\bullet}$).[45-50] In carboxymethylated cyt *c*, Met80 was carboxymethylated and the Met80−heme iron bond was cleaved.[30] Thus, carboxymethylated cyt *c* reacted with excess hydrogen peroxide, generating a new absorption band at 628 nm. This band was characteristic of the Compound I-like species, which was stable for more than 30 min.[30] Compound I-like species of cyt *c* (~650 nm) has also been observed during the reaction of dimeric cyt *c* with *meta*-chloroperbenzoic acid.[28] The absorption band around 650 nm, characteristic of Compound I, has also been detected in other heme proteins, such as HRP (~622 and ~650 nm).[45,46] myoglobin (Mb) (648 nm),[47] and a dye-decolorizing peroxidase (613 and 648 nm).[48] In addition, the Compound I species has been reported to be stabilized in the distal heme pocket mutants of Mb, and an absorption band was observed at 648 nm.[49,50] Therefore, the oxy complex of Δ8384 cyt *c* was presumably converted to the Compound I-like species by further incubation with DTT under air, where Met80 of Δ8384 cyt *c* was site-specifically oxidized by the Compound I-like species.

After incubation of Met80-unmodified ferric Δ8384 cyt *c* in the presence of 500 μM DTT under air for 3 h, a new peak was observed at m/z = 12,093 in its MALDI-TOF mass spectrum, in addition to the peak at m/z = 12,077 (See ESI, Fig. S1†). The mass value at m/z = 12,093 corresponded well to that of cyt *c* oxidized with an oxygen atom. The incubated Δ8384 cyt *c* was also digested with lysyl endopeptidase and the obtained fragments were investigated by mass spectroscopy. The peptide fragment with the mass of m/z = 667.4 was detected in the HPLC chromatogram, and the mass peaks corresponding to $[IFIK]^+$ (m/z: 520.9, Mm: 520.4), $[FIK]^+$ (m/z: 407.2, Mm: 407.3), $[IK]^+$ (m/z: 260.1, Mm: 260.2), $[K]^+$ (m/z: 147.1, Mm: 147.1), and the immonium ion with an additional oxygen atom (m/z: 120.1, Mm: 120.1) were all observed in the tandem mass spectrum of the peptide fragment of m/z = 667.4 (See ESI, Fig. S2†). These results supported the interpretation that non-coordinating Met80 of Δ8384 cyt *c* was oxidized during incubation under air in the presence of DTT. By incubation of Δ8384 cyt *c* under air at 25 °C for 3 h in the presence of another reducing reagent (tris(2-carboxyethyl)phosphine, TCEP) instead of DTT, the intensity of the peak at m/z = 12094



 

increased significantly in the MALDI-TOF mass spectrum, where its intensity was higher than that of the m/z = 12078 peak of the unmodified protein (See ESI, Fig. S3†). These results clearly show that modification of Δ8384 cyt *c* by incubation under air in the presence of a reducing reagent was not specific to DTT.

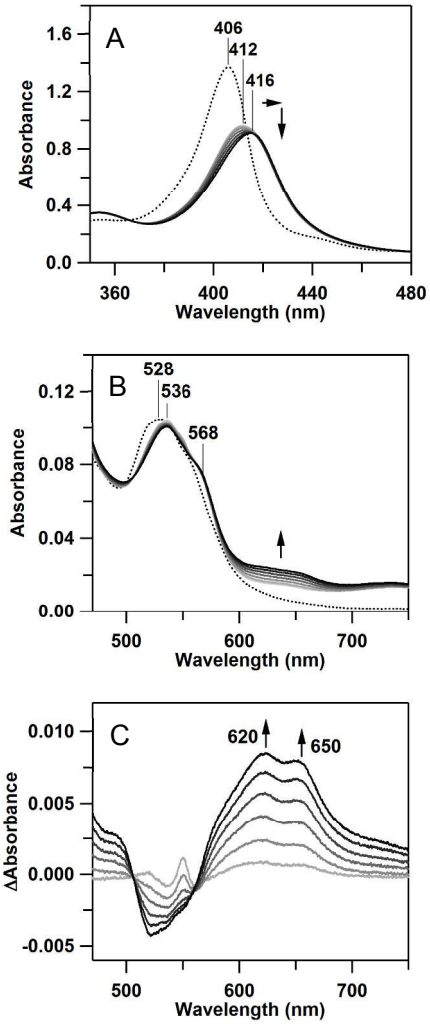


**Fig. 8** Absorption spectral changes of ferric Met-unmodified Δ8384 human cyt *c* by incubation with DTT under air. (A) Soret region. (B) Q-band region. The dotted lines represent the spectra of ferric Δ8384 cyt *c*. The spectra were measured after incubation with DTT for 0, 30, 60, 90, 120, 150, and 180 min. (C) Difference spectra in the Q-band region. The difference spectra were obtained by subtraction of the spectra measured at 30, 60, 90, 120, 150, and 180 min after the DTT addition with the initial spectrum measured after the DTT addition. Measurement conditions: Δ8384 cyt *c* concentration, 12 μM; DTT concentration, 500 μM; buffer, 50 mM potassium phosphate buffer; pH, 7.0; temperature, 25 ºC.

We propose the following mechanism for Met80 oxidation in cyt *c* (Fig. 9). Met80 dissociates from the heme iron in cyt *c* by interaction with CL.[23] A reducing agent reduces the Met-dissociated heme iron and allows molecular oxygen to bind to the reduced heme, forming the oxy complex. When an additional electron is supplied to the oxy complex, a presumably high-valent ferryl-oxo species (Compound I) forms and oxidation of Met80 occurs, which is analogous to the mechanism of cytochrome P450.[51-53] These results show that a self-catalytic oxidation of cyt *c* at Met80 occurs by its heme and molecular oxygen when Met80 dissociates from the heme iron in a reducing environment.

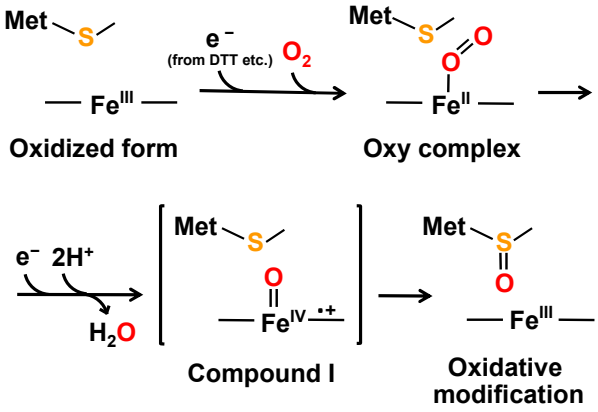


**Fig. 9** Putative mechanism for Met80 oxidation of cyt *c* by reaction with molecular oxygen initiated by Met80 dissociation from the heme iron.

Met80 of cyt *c* is reported to dissociate from the heme iron by interaction with CL in the inner membrane of mitochondria,[23,54] where molecular oxygen is reduced to water by cytochrome *c* oxidase. The present results demonstrate that cyt *c* may also react with molecular oxygen when it is reduced at the intermembrane space. The ferrous cyt *c*:CL:$O_2$ complex may accept another electron in mitochondria to form Compound I analogous to the oxidation route used by cytochrome P450.[51,53] Oxidation of Met80 by Compound I in cyt *c* may alter the heme environment and reduce the redox potential of cyt *c*,[55] causing increase in peroxidase activity of cyt *c* and permeabilization of the mitochondrial outer membrane.[56] It has been reported that CL oxidation may occur by the reaction of ferrous cyt *c*:CL complex with molecular oxygen similar to the reaction of cytochrome P450,[51,54,57] in addition to the reaction of ferric cyt *c*:CL complex with a peroxide similar to the reaction of peroxidases.[54,58] In fact, oxidation of CL has been suggested to initiate apoptosis by releasing cyt *c* from mitochondria to cytosol.[16,17,25,26] The modification of cyt *c* did not depend on the reducing reagent, DTT or TCEP. In addition, the modification of Δ8384 cyt *c* occurred during purification from *E. coli* under air but not when using $N_2$-bubbled buffer. These results suggest that the reducing reagents in the biological system may reduce the heme allowing molecular oxygen to bind to the heme, and modification of cyt *c* may occur in biological systems when Met80 is dissociated from the heme iron. However, further experiments are necessary to elucidate the reaction in more detail.

### Conclusions

Ferric human cyt *c* was modified with incorporation of an oxygen atom by reaction with molecular oxygen in the presence of DTT and CL-containing liposomes under air, presumably due to Met80 dissociation from the heme iron. To systematically study the effect of Met80 dissociation on the modification of cyt *c*, we constructed the Δ8384 cyt *c* mutant as a model of the cyt *c*:CL complex by removal of Val83 and Gly84 from its loop, without changing the secondary structures significantly. Δ8384 human cyt *c* was modified when purified in the presence of molecular oxygen, whereas not in the absence of molecular oxygen. Tandem mass measurements showed that Met80 of Δ8384 cyt *c* was site-specifically modified to methionine sulfoxide by reaction with molecular oxygen in the presence of DTT, without modification of other residues. The Met80 modification of cyt *c* by molecular oxygen occurred through formation of the oxy and Compound I-like species, similar to the oxidation reaction catalyzed by cytochrome P450.

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