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COMMUNICATION

Peroxidase activity enhancement of horse cytochrome *c* by dimerization†

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The peroxidase activity of horse cytochrome *c* was enhanced by its dimerization, where its Compound III (oxy-form) and Compound I (oxoferryl porphyrin π-cation radical) species were detected in the reactions with hydrogen peroxide and *meta*-chloroperbenzoic acid, respectively. These results show that oligomeric cytochrome *c* can contribute as a proapoptotic conformer by the increased peroxidase activity.

Introduction

Cytochrome *c* (cyt *c*) is a well-known hemoprotein characterized by the covalent attachment of the heme to the sequence motif of CXXCH *via* two thioether bonds. The histidine residue serves as the fifth axial ligand of cyt *c*, whereas the sixth ligand is commonly a methionine.[1–3] Cyt *c* transfers electrons from the respiratory Complex III to Complex IV in mitochondria,[1] and is also involved in initiation of apoptosis upon its release to the cytoplasm. The peroxidase activity of cyt *c* is increased by the hydrophobic interaction with cardiolipin (CL). The peroxidase activity of cyt *c* results in the oxidation of CL and subsequently in the execution of apoptosis *via* permeabilization of the mitochondrial membrane and release of proapoptotic factors from mitochondria.[4]

For nearly half a century, it has been known that monomeric cyt *c* can be converted into polymeric forms by incubation with ethanol or trichloroacetic acid.[5] Recently, we have solved the X-ray crystal structures of dimeric (PDB ID: 3NBS) and trimeric cyt *c* (PDB ID: 3NBT) and found that Met80 dissociates from the heme iron in the oligomers.[6] The dissociation of Met80 creates a binding site to the heme for a substrate and an oxidant, influencing the peroxidase activity of cyt *c* in the oligomers. This influence on the peroxidase activity may be related to the initiation of apoptosis. However, the peroxidase activity of oligomeric cyt *c* including dimeric cyt *c* has not been studied in detail. Bearing this in mind prompted us to study the peroxidase activity of oxidized dimeric cyt *c* using hydrogen peroxide and *meta*-chloroperbenzoic acid (*m*CPBA).

Results and discussion

To evaluate the influence of oligomerization on the peroxidase activity of cyt *c*, steady-state kinetics were measured with two commonly used substrates, 2-methoxyphenol (guaiacol) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), for their oxidations by monomeric and dimeric cyt *c* in the presence of H_2O_2 in 50 mM potassium phosphate buffer, pH 7.0. The rates of product formation of guaiacol and ABTS oxidations were determined from the increases in the absorbances at 470 (ε_{470} = 26.6 $mM^{-1}cm^{-1}$)[7,8] and 730 nm (ε_{730} = 14 $mM^{-1}cm^{-1}$),[9] respectively. The steady-state rates were obtained from the maximum of the first derivative of the product formation curves.[7,10] Steady-state rates at various concentrations of guaiacol or ABTS were plotted, and the kinetic parameters were obtained by fitting the data to the Michaelis–Menten equations, where substrate inhibition was considered at high substrate concentration in ABTS oxidation (Fig. 1).[11] The obtained kinetic parameters are summarized in Table 1.

In the Michaelis–Menten equation, the Michaelis constant (K_m) is a measure of the affinity of the enzyme for the substrate, where a lower K_m value indicates a stronger substrate binding. The turnover number (k_{cat}) represents the rate constant of the chemical process, and the k_{cat}/K_m value indicates the efficiency of the enzyme toward the substrate (overall catalytic activity). The K_m value of dimeric cyt *c* was slightly higher than that of monomeric cyt *c* for guaiacol oxidation, whereas the k_{cat} and k_{cat}/K_m values of dimeric cyt *c* were about 4–5 fold higher than those of the monomer. Similar results were obtained for ABTS oxidation. The K_m, k_{cat} and k_{cat}/K_m values for the dimer were about 2-, 6- and 4-fold higher, respectively, than those for the monomer. We attribute the improved overall peroxidase activity of cyt *c* to the dissociation of Met80 in the dimer,[6] since it has been reported that the peroxidase activity of cyt *c* increases by the rupture of the Met80–heme iron bond.[10,12]

Interestingly, the K_m values for both guaiacol and ABTS oxidations by monomeric and dimeric cyt *c* (5–15 μM) were much lower than those of other hemoproteins, such as horseradish peroxidase (HRP) (K_m values are 1.4 and 0.22 mM for guaiacol and ABTS, respectively)[13] and myoglobin (K_m is 54 mM for guaiacol oxidation).[14] These substrates may not bind at the heme pocket of cyt *c*, and the oxidation occurs either at the heme edges

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Table 1 Kinetic parameters of guaiacol and ABTS oxidations[a]

	Guaiacol			ABTS		
Cyt *c*	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
Monomer	0.13	11.0	12 000	1.77	5.5	320 000
Dimer	0.66	14.2	47 000	11.3	9.4	1 200 000

[a] Reaction conditions: 1 μM protein (heme unit) and 50 mM H_2O_2 in 50 mM potassium phosphate buffer, pH 7.0, at 25 °C. All the k_{cat} and K_m values are averages of three measurements with an error less than ±5%.

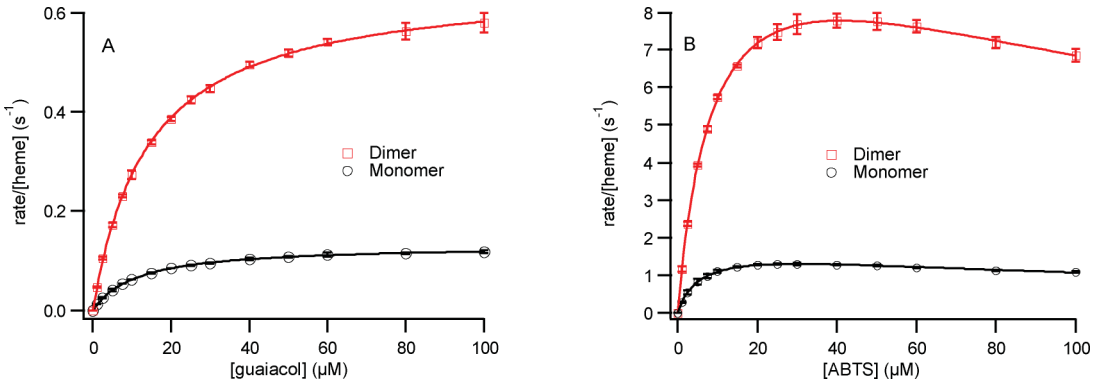

Fig. 1 Steady-state rates of monomeric (○) and dimeric (□) cyt *c*-catalyzed oxidations as functions of substrate concentrations: guaiacol (A) and ABTS (B). Reaction conditions: 1 μM protein (heme unit), 50 mM H_2O_2, 50 mM potassium phosphate buffer, pH 7.0, 25 °C. The curves obey the following equations: $v/[\text{protein}] = k_{cat}\cdot[\text{guaiacol}]/(K_m+[\text{guaiacol}])$ and $v/[\text{protein}] = k_{cat}\cdot[\text{ABTS}]/(K_m+[\text{ABTS}]+[\text{ABTS}]^2/K_i)$. Substrate inhibition appeared at high concentration of ABTS with $K_i = 168 \pm 7$ μM and 178 ± 7 μM for monomeric and dimeric cyt *c*, respectively.

as for HRP[15] or on the protein surfaces as for cytochrome *c* peroxidase.[16]

It is noteworthy that the K_m values of guaiacol and ABTS oxidations by dimeric cyt *c* are slightly higher than those by monomeric cyt *c*. These results show that the open heme pocket in dimeric cyt *c* by Met80 dissociation does not contribute to facilitating access by the substrates. Indeed the structural change in cyt *c* upon dimerization by domain swapping of the C-terminal helix results in covering the protein surface close to the heme.[6] This structural change presumably disturbs the surface accessibility for the substrates and causes weaker binding for guaiacol as well as for ABTS. Therefore, the enhanced overall peroxidase activity of dimeric cyt *c* is obtained not by the improvement of substrate access, but the facilitation of the actual chemical process.

To disclose the origin of the increase in the k_{cat} value in dimeric cyt *c*, we investigated the reaction intermediates by transient optical absorption spectra in the absence of an organic substrate. The initial intermediate known as Compound I (oxoferryl porphyrin π-cation radical) is formed in the reaction of peroxidases.[17,18] For the detection of Compound I in the peroxidase reaction of cyt *c*, the reactions of ferric dimeric cyt *c* with H_2O_2 were followed by a stopped-flow rapid-scan system. When a low concentration of dimeric cyt *c* (5 μM, heme unit) reacted with H_2O_2 (50 mM) at 25 °C, disappearance of the Soret absorbance was observed (data not shown), indicating protein degradation due to the self-oxidation of the heme porphyrin ring.[19] These results are consistent with the previous studies[20,21] and show that Compound I of cyt *c* is very difficult to detect due to its extremely high reactivity and low steady-state concentration. To accumulate higher steady-state concentrations of Compound I, reactions of high concentrations of ferric dimeric cyt *c* up to 50 μM (heme unit) with high concentrations of H_2O_2 up to 1 M were performed at 25 °C. At high concentrations of dimeric cyt *c* and H_2O_2, two new bands at ~540 and ~570 nm were detected in the transient absorption spectra (Fig. 2). The spectrum resembled the absorption spectrum of the oxygen-bound form of the Met80Ala mutant of cyt *c* (537 and 570 nm).[22] The spectrum was also similar to those of oxymyoglobin and Compound III (oxy-form) of HRP, which was observed by placing the ferric HRP crystal in H_2O_2 solution.[23]

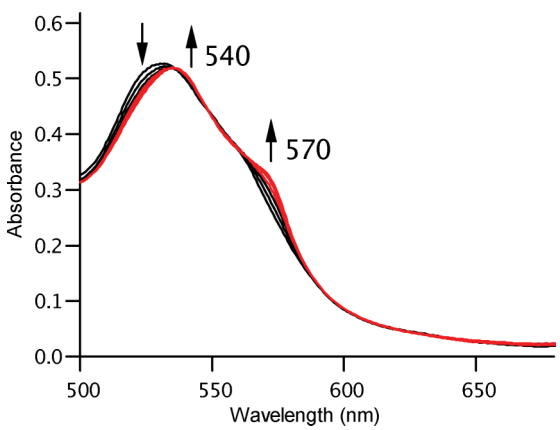

Fig. 2 Absorption spectral changes in the reaction of dimeric cyt *c* with H_2O_2 observed by stopped-flow rapid-scan measurements. Spectra at 0, 15, 25, 35, 50 and 75 ms after deadtime (deadtime is about 1 ms) are shown. Spectra at 0, 15 and 25 ms are shown in black. Conditions: 50 μM dimeric cyt *c* (heme unit); 1 M H_2O_2; 50 mM potassium phosphate buffer, pH 7.0; 25 °C.

Therefore, the transient species exhibiting new absorption bands at ~540 and ~570 nm is assigned to Compound III of dimeric cyt *c*.[23] No significant difference was observed in the absorption spectral change of dimeric cyt *c* when the reaction with H_2O_2 was performed under anaerobic conditions, indicating that the heme-bound oxygen of Compound III was produced from H_2O_2 during the reaction. In fact, the new absorption bands were not observed in the absorption spectra when ferrous dimeric cyt *c* was reacted with dioxygen. Similar spectral changes were detected in the reaction of monomeric cyt *c* with H_2O_2 under similar conditions, but the spectral changes were much slower than those of dimeric cyt *c* due to coordination of Met80 to the heme iron in monomeric cyt *c*. These results suggest that Compound I generated in the reaction of cyt *c* with H_2O_2 is rapidly converted to Compound III under a large excess of H_2O_2.

In an attempt to circumvent the conversion of Compound I to Compound III in cyt *c*, *m*CPBA was used as the oxidant instead of H_2O_2. By incubation of 50 μM oxidized dimeric cyt *c* with 4.0 mM *m*CPBA at 25 °C, a broad absorption band at 570–700 nm was detected with a decrease in the Q band at ~530 nm, where an isosbestic point was observed at ~565 nm (Fig. 3). To further investigate the spectral change in detail, we calculated the differential spectra based on the initially collected spectrum, where the difference spectra exhibited two absorption bands around 595 and 650 nm (Inset of Fig. 3). The spectrum of the intermediate species in cyt *c* resembles those of Compound I of HRP and the π-cation radical of the one-electron-oxidized Zn tetraphenylporphyrin complex (ZnTPP).[23–26] The visible band around 650 nm is characteristic of Compound I observed for other hemoproteins such as myoglobin and HRP.[27,28] Therefore, the absorption increase in the region of 570–700 nm is assigned to the formation of Compound I of cyt *c*. The formation of Compound I was also observed by the reaction of monomeric cyt *c* with *m*CPBA under the same conditions, although the formation was slower.

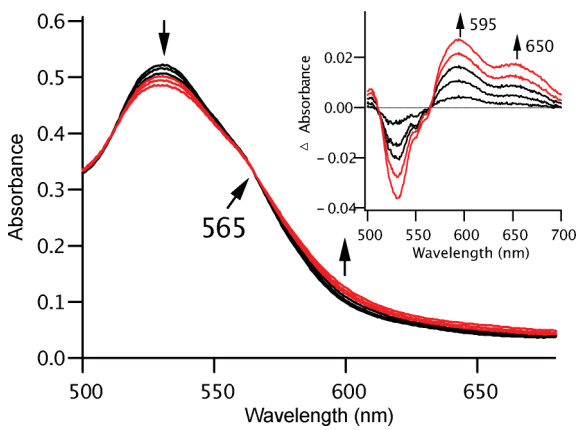

Fig. 3 Absorption spectral changes in the reaction of dimeric cyt *c* with *m*CPBA observed by stopped-flow rapid-scan measurements. Spectra at 0, 5, 20, 30, 45 and 70 ms after deadtime are shown. Spectra at 0, 5 and 20 ms are shown in black. Inset: Difference spectra of each spectrum subtracted with the spectrum at 0 ms. Conditions: 50 μM dimeric cyt *c* (heme unit); 4 mM *m*CPBA; 50 mM potassium phosphate buffer, pH 7.0; 25 °C.

To evaluate the effect of Compound I in cyt *c* on the oxidation rate of the substrates, formation of Compound I and cyt *c*-catalyzed oxidation of guaiacol were examined in the presence of 2.5 mM *m*CPBA in 50 mM potassium phosphate buffer, pH 7.0, at 25 °C. The formation rate of Compound I was estimated by least-square fitting the absorbance increase at 595 nm to a single exponential function. The formation rate constant of Compound I of dimeric cyt *c* was 0.019 s^{-1}, which was 6-fold higher than that of the monomer (0.0032 s^{-1}) (ESI† , Fig. S1 and Table S1). The oxidation rate of guaiacol catalyzed by dimeric cyt *c* was ~6-fold higher than that by the monomer, which corresponded very well to the difference in the formation rate of Compound I. These results show that formation of Compound I is the rate determining step in the peroxidase reaction cycle of cyt *c*. In the case of Compound I formation in monomeric cyt *c*, dissociation of the axial ligand Met80 precedes H_2O_2 or *m*CPBA binding, whereas H_2O_2 and *m*CPBA can bind easily to the heme iron in dimeric cyt *c*. Therefore, the enhanced peroxidase activity of dimeric cyt *c* is mainly due to its fast formation of Compound I owing to an opened ligand-binding space at the heme site.

Cyt *c* has been reported to change its tertiary structure with cleavage of the Met80-heme iron bond by interaction with CL in mitochondria membrane and, in turn, increase the cyt *c* peroxidase activity leading to apoptosis.[4,29] By dimerization, the tertiary structure of cyt *c* changes with dissociation of Met80,[6] and the peroxidase activity increases. These results indicate that oligomerization of cyt *c* can lead to tertiary structural changes with enhanced peroxidase activity contributing as a proapoptotic conformer.

Conclusions

We prepared dimeric horse cyt *c* and studied its peroxidase activity. Our studies show that the peroxidase activity of cyt *c* is enhanced by dimerization due to the faster formation of Compound I, since the cavity at the heme site is more open than in the monomer.

Experimental section

Preparation of dimeric cyt *c*

The cyt *c* dimer was prepared by dissolving about 100 mg of horse ferric cyt *c* (Wako, Osaka, Japan) in 10 ml of 50 mM potassium phosphate buffer, pH 7.0, followed by addition of ethanol to 60% (v/v). The cyt *c* solution was centrifuged and the precipitate was lyophilized. The lyophilized precipitate was then dissolved in 10 ml of 50 mM potassium phosphate buffer, pH 7.0. After incubation at 37 °C for 1.5 h, the cyt *c* oligomer solution was filtrated, and dimeric cyt *c* was separated and purified by gel chromatography (HiLoad 26/60 Superdex 75, GE Healthcare) with 50 mM potassium phosphate buffer, pH 7.0, at 4 °C. (ESI†, Fig. S2)

Measurements of peroxidase activity

The catalytic steady-state kinetics of 2-methoxyphenol (guaiacol) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) oxidations in the presence of hydrogen peroxide were studied using an RSP-601 stopped-flow apparatus equipped with a photomultiplier tube detector (Unisoku, Osaka, Japan) at 25 °C. A H_2O_2 solution was prepared from its 30% stock solution, and the H_2O_2 concentration was determined with the absorption coefficient of 43.6 M^{-1} cm^{-1} at 240 nm.[30]

A monomeric or dimeric cyt *c* (2.0 μM, heme unit) solution with various concentrations of guaiacol or ABTS (0–200 μM) in 50 mM potassium phosphate buffer, pH 7.0, and the H_2O_2 (100 mM) solution in the same buffer were preincubated at 25 °C for 5 min. After the preincubation, both solutions were mixed together in the mixing cell of the stopped-flow apparatus to start the oxidation reaction. The steady-state reaction rates of guaiacol and ABTS oxidations were obtained by monitoring the absorbance increases at 470 and 730 nm using molar absorption coefficients of 26.6[7,8] and 14 $mM^{-1}cm$,[9] respectively. Each experiment was repeated at least 3 times.

The product formation curves of guaiacol and ABTS oxidations catalyzed by horse heart monomeric and dimeric cyt *c* were similar to those of guaiacol oxidation catalyzed by *Paracoccus versutus* cytochrome *c*-550[7] and yeast iso-1 cytochrome *c*.[10] The product formation curves exhibited an initial activation phase (I) before a linear phase (II), followed by a decrease in activity (III) and finally a decrease in absorption. The rate of the steady-state reaction was determined from the maximum of the first derivative of the product formation curve, *i.e.*, the linear phase (ESI†, Fig. S3).

Reactions of cyt *c* with hydrogen peroxide

All reactions of ferric cyt *c* with hydrogen peroxide were carried out in 50 mM potassium phosphate buffer, pH 7.0. The spectral changes were monitored by an RSP-601 stopped-flow apparatus equipped with a photodiode array detector (Unisoku). The cyt *c* solution of various concentrations (10–100 μM, heme unit) and the H_2O_2 solution of various concentrations (0.1–2 M) were preincubated separately at 25 °C for 5 min. After the preincubation, both solutions were mixed together in the mixing cell of the stopped-flow apparatus.

Reactions of cyt *c* with *meta*-chloroperbenzoic aicd (*m*CPBA)

All reactions of ferric cyt *c* with *m*CPBA were carried out in 50 mM potassium phosphate buffer, pH 7.0. Reactions were carried out by mixing the solution of monomeric or dimeric cyt *c* (100 μM, heme unit) with the *m*CPBA solution (5 mM) using the RSP-601 stopped-flow apparatus (Unisoku) at 25 °C. Pseudo first-order formation rates of the Compound I species of monomeric and dimeric cyt *c* were determined by least-square fitting the absorbance increases at 595 nm to a single exponential function (ESI†, Fig. S1 and Table S1). Spectral changes were monitored by a photodiode array detector (Unisoku).

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