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# Reduction of plastocyanin by tyrosine-containing oligopeptides

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**Abstract**

Oxidized plastocyanin (PC) was reduced with TyrTyrTyr and LysLysLysLysTyrTyrTyr (KKKKYYY) oligopeptides at neutral pH. The TyrTyrTyr site of the peptides provided an electron to the copper active site of PC, whereas the tetralysine site of KKKKYYY functioned as the recognition site for the negative patch of PC. The reciprocal initial rate constant ($1/k_{int}$) increased linearly with the reciprocal TyrTyrTyr concentration and proton concentration, although the electron transfer rate decreased gradually with time. The results showed that PC was reduced by the deprotonated species of TyrTyrTyr. A linear increase of log $k_{int}$ with increase in the ionic strength was observed due to decrease in the electrostatic repulsion between negatively charged PC and deprotonated $(TyrTyrTyr)^-$. PC was reduced faster by an addition of KKKKYYY to the PC–TyrTyrTyr solution, although KKKKYYY could not reduce PC without TyrTyrTyr. The ESI-LCMS spectrum of the products from the reaction between PC and TyrTyrTyr showed molecular ion peaks at $m/z$ 1015.7 and 1037.7, which suggested formation of a dimerized peptide that may be produced from the reaction of a tyrosyl radical. The results indicate that PC and the tyrosine-containing oligopeptides form an equilibrium, $PC_{ox}/(oligopeptide)^- \leftrightarrows PC_{red}/(oligopeptide)^{\bullet}$. The equilibrium is usually shifted to the left, but could shift to the right when the produced oligopeptide radical reacts with unreacted peptides. For the reaction of PC with KKKKYYY in the absence of TyrTyrTyr, the produced KKKK(YYY)$^{\bullet}$ radical peptide could not react with other KKKKYYY peptides, since they were positively charged. In the presence of both KKKKYYY and TyrTyrTyr, PC may interact effectively with KKKKYYY through its tetralysine site and receive an electron from its TyrTyrTyr site, where the produced KKKK(YYY)$^{\bullet}$ may interact with TyrTyrTyr peptides.


*Keywords:* Plastocyanin; Tyrosine-containing peptide; Lysine peptide; Protein–peptide interaction; Active site reduction

## 1. Introduction

Oxidation of tyrosine is frequently observed in proteins [1–5], and concomitant electron transfer is often coupled with the reduction of the metal site, which is indispensable for the enzymatic reaction. Oxidation of tyrosine produces a tyrosyl radical, which is highly reactive towards phenols [6]. For example, cross-linking tyrosines are formed to produce a hard fertilization membrane by oxidation of protein-bound tyrosyl residues in the presence of peroxidase [7–10]. These tyrosine-containing peptides have also been shown to polymerize by oxidation with compound I or compound II of horseradish peroxidase [11–14], lactoperoxidase [13], and myeloperoxidase [15,16]. Formation of *o*-tyrosine and 3,3′-dityrosine in proteins by metal-catalyzed oxidation with $Cu^{II}$ and $H_2O_2$ has been observed, where dityrosine has been shown to accumulate in the protein upon the oxidation [17]. Oxidation of a tyrosine

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residue to a quinone by post-translational modification has also been observed in amine oxidase [18,19]. Since tyrosine oxidation may occur in proteins, it is important to understand the nature of the tyrosine oxidation process in proteins. Studies on the reactions of tyrosine-containing peptides with metalloproteins at neutral pH would provide such information.

Plastocyanin (PC) is a mobile copper protein existing in the thylakoid lumen of photosynthetic organisms. PC accepts an electron from cytochrome *f* (cyt *f*), a subunit of the cytochrome $b_6f$ complex, and donates it to the reaction center chlorophyll in the photosystem I (PSI) complex [20–22]. PC is classified as a Type 1 copper protein, which exhibits a low energy ligand-to-metal charge transfer band near 600 nm in the absorption spectra and a narrow hyperfine coupling constant ($|A_{\|}| < 90 \times 10^{-4}\ \mathrm{cm}^{-1}$) in the electron paramagnetic resonance spectra [23,24]. Plant PC contains one copper atom with two histidine nitrogen atoms, one methionine sulfur atom, and one cysteine sulfur atom coordinated in a distorted tetrahedral geometry, which is revealed by the crystal structures of PC [25–29].

Plant PC usually possesses two highly conserved sites which have been considered as molecular recognition sites for its redox partners, cyt *f* and PSI: One site is located at the solvent-accessible site containing the Cu-coordinated histidine (Cu-adjacent hydrophobic patch), and the other site is positioned at another solvent-accessible site including several acidic residues (Cu-remote negative patch) (Fig. 1). The Cu-remote negative patch of PC consists of two clusters: One lower cluster (Asp42/Glu43/Asp44/Glu45) and another upper cluster (Glu59/Glu60/Asp61) (Fig. 1). Both of these clusters have been indicated to be essential for the binding of PC to cyt *f* [30] and have been shown to interact with charged molecules and proteins [31,32]. The negative patch of PC and the positively charged site of cyt *f* interact through electrostatic interactions to form a PC–cyt *f* complex for electron transfer [33–43], whereas the hydrophobic patch of PC has also been shown to be crucial for the PC–cyt *f* complex by NMR and mutation studies [30,44–47]. It has been shown with the use of photoinduced zinc cytochrome *c* (cyt *c*) that PC and cyt *f* or cyt *c* react with each other in different configurations resulting from the protein–protein interaction termed as the gating process for electron transfer [48–50].

We have previously shown that oxidized cyt *c* can be reduced with a tyrosine-containing peptide, tyrosyltyrosylphenylalanine (TyrTyrPhe), producing an oxidized species of the tyrosine [51]. From the mass spectra of the reaction products, formation of quinone and tyrosine derivatives of the peptide was suggested. We proposed formation of a cyt $c_{ox}$/(TyrTyrPhe)$^-$ ⇆ cyt $c_{red}$/(TyrTyrPhe)$^{\cdot}$ equilibrium, which is usually shifted to the left but shifts to the right by the interaction of the tyrosyl radical with unreacted TyrTyrPhe peptides though hydrophobic interaction. It would be important to check whether tyrosine-containing peptides reduce other metalloproteins, to elucidate the mechanism of metalloprotein reduction by the peptides, and to design a more reactive peptide. We, therefore, investigated the reaction of oxidized PC with TyrTyrTyr and LysLysLysLysTyrTyrTyr (KKKKYYY), which possesses a molecular interaction site for PC. The present work shows that metalloproteins could be reduced effectively by tyrosine-containing oligopeptides.

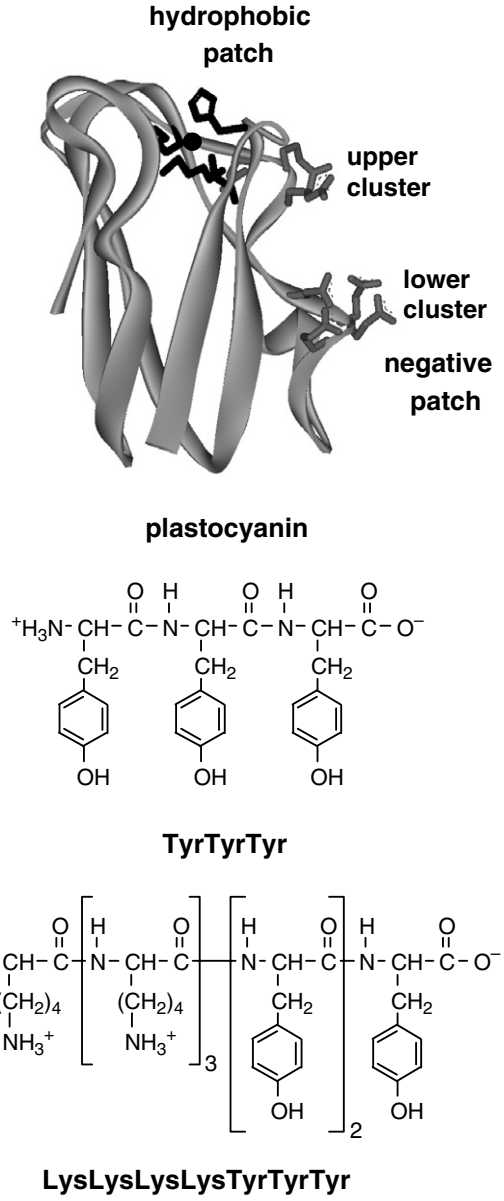


Fig. 1. Protein structure of silene PC (PDB entry, 1BYO) and chemical structures of the peptides used in this study.

## 2. Experimental

### 2.1. Sample preparation

*Silene pratensis* (white campion) PC was purified as described before [32,33]. Purified PC was dialyzed with 1 or 20 mM phosphate buffer, pH 6.4–7.5, before each measurement, and the protein concentration was adjusted by

the absorption spectrum. The TyrTyrTyr peptide was purchased from Bachem (Budendorf, Switzerland), and KKKKYYY and AspAspAspAspTyrTyrTyr (DDDDYYY) peptides were purchased from Toray Research Center (Kanagawa, Japan). The purity of each oligopeptide was checked by the MALDI-TOF mass measurement and the elemental analysis. The TyrTyrTyr peptide was first dissolved in 1 or 20 mM phosphate buffer of the measuring pH with the peptide concentration a little more than 4 mM. The pH value and peptide concentration were readjusted to the desired pH and 4 mM, respectively, by using 1 or 20 mM phosphate buffer and a small amount of 1 or 20 mM phosphate buffer containing 1 or 0.1 M NaOH. For the measurement with different ionic strength, NaCl was added to the protein solution before mixing it with the peptide solution. Each PC and TyrTyrTyr solution was filtrated before use.

### 2.2. Kinetic measurements

PC (50 μM) in 1 or 20 mM phosphate buffer, pH 6.4–7.5, was mixed with TyrTyrTyr in the same buffer at 15 °C under air, nitrogen atmosphere, and oxygen atmosphere. To investigate the effect of the KKKKYYY peptide, the PC solution was mixed with the TyrTyrTyr solution containing KKKKYYY. The absorption change was recorded with a Shimadzu UV-2450PC spectrophotometer. The initial rate constant was calculated by dividing the initial slope of the absorption change at 600 nm with its initial absorbance.

### 2.3. Identification of the reaction products from the peptide

PC (50 μM) in 20 mM phosphate buffer, pH 7.0, was mixed with TyrTyrTyr (1 mM) in the same buffer at 20 °C. The solution was placed under ice after 1 h of reaction. After the reaction, the solution (50 μL) was mixed with solvent A (0.1% TFA-$H_2O$) (20 μL) and filtrated. The filtrated solution (20 μL) was purified with a HPLC system (Waters, 600E/996) using a C18 column (Wakosil-II 5C18 250 × 4.0 mm): Flow rate, 0.8 mL/min; detection, absorption at 280 and 600 nm; mobile phase (after loading the sample), solvent A for 5 min, a linear 40-min gradient from 0 to 100% solvent B (0.1% TFA-$CH_3CN$) in solvent A, and solvent B for 5 min were performed. The fractions were collected manually. The mass spectra of the collected peptides were measured with an ESI-LCMS mass spectrometer (Shimadzu Corporation, LCMS-2010EV, Kyoto, Japan).

## 3. Results

### 3.1. Absorption changes of oxidized PC by interaction with TyrTyrTyr

The band at 597 nm in the absorption spectrum of oxidized PC is assigned to the cysteine thiolate ($S_{cys}$)-to-Cu(II) charge transfer band, which is typical for the oxidized form. The intensity of this 597-nm band decreased by the reaction with TyrTyrTyr at pH 7.0 under air (Fig. 2a), where the absorption changes occurred with an isosbestic point at about 430 nm. By an addition of ferricyanide to the solution after the reaction, the intensity of the absorption band at 597 nm recovered. These absorption changes clearly show that PC was reduced by TyrTyrTyr. The reduction rate constant decreased gradually with time (Fig. 2b), and the reduction rate of PC was much slower for the reaction with tyrosinol or TyrTyr than that with TyrTyrTyr under the same condition. These properties were similar to those observed for the interaction of cyt *c* with tyrosine-containing peptides [51].

The absorption around 350 nm increased by the reaction of oxidized PC with TyrTyrTyr, where the absorption increase was attributed to formation of the reaction products. Since the product exhibited an absorption band at a longer wavelength than TyrTyrTyr, formation of a more conjugated species than TyrTyrTyr was suggested. The 350-nm absorption band, however, was difficult to detect

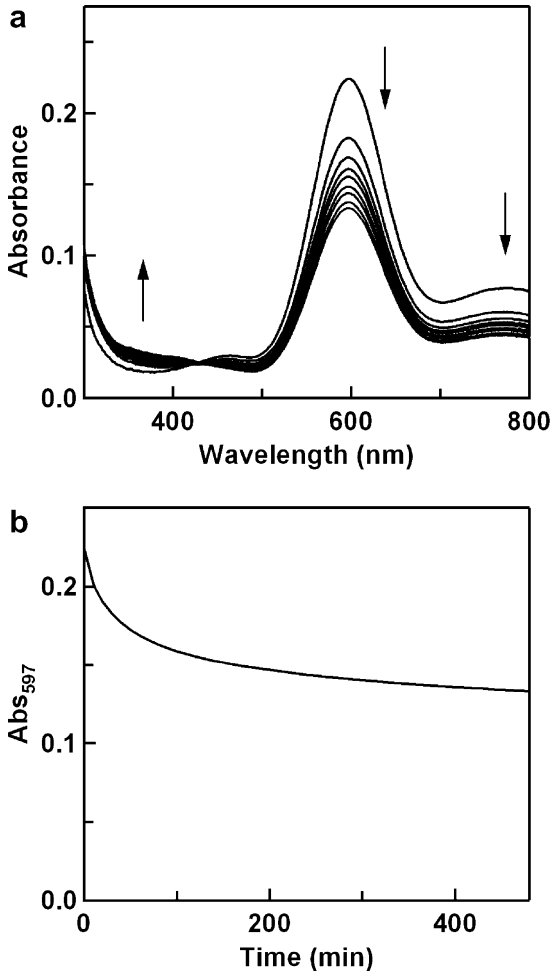


Fig. 2. Absorption change of oxidized PC by interaction with TyrTyrTyr. (a) Absorption spectra after reaction of 0-, 30-, 60-, 90-, 120-, 180-, 240-, 360-, and 480-min are shown. (b) Time course of the absorption change at 597 nm. Experimental conditions: PC, 50 μM; TyrTyrTyr, 2.0 mM; phosphate buffer (20 mM), pH 7.0; 15 °C.

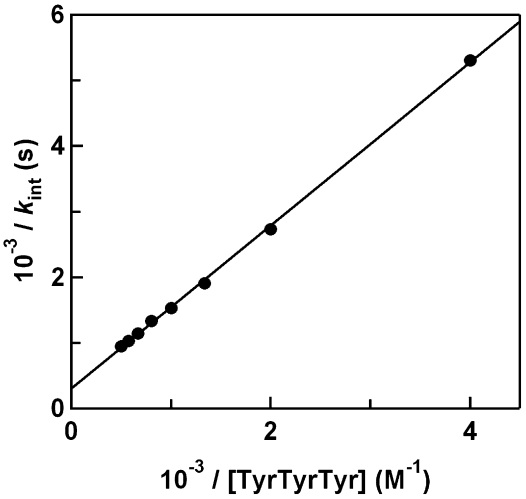


Fig. 3. Plots of $1/k_{int}$ vs the reciprocal TyrTyrTyr concentration, together with the least-squares-fitted line according to Eq. (4). Experimental conditions: PC, 50 μM, TyrTyrTyr 0.25–2.0 mM; phosphate buffer (20 mM), pH 7.0; 15 °C.

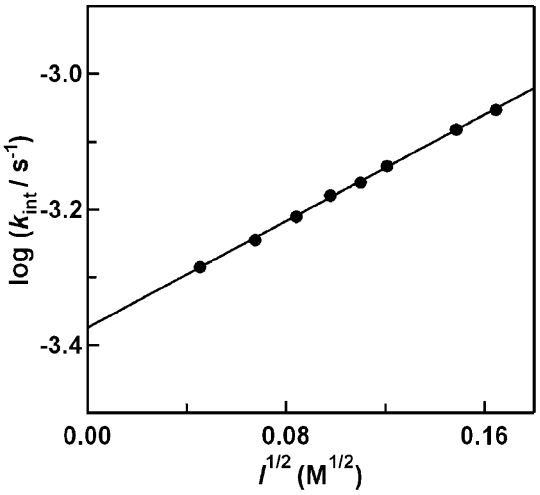


Fig. 5. Plots of log $k_{int}$ vs root of the ionic strength, together with the least-squares-fitted line according to Eq. (6). Experimental conditions: PC, 50 μM; TyrTyrTyr, 1.0 mM; phosphate buffer (1 mM), pH 7.0, with NaCl (0, 2.5, 5.0, 7.5, 10, 12.5, 20, and 25 mM); 15 °C.

previously in the reaction of cyt *c* with TyrTyrPhe due to the overlapping of this band with the absorption band of cyt *c*.

Since the reaction rate constant decreased gradually with time (Fig. 2b), we measured the initial rate constant ($k_{int}$) of the reaction. The reciprocal initial rate constant ($1/k_{int}$) increased linearly with a slope of 1.2 M s against the reciprocal TyrTyrTyr concentration (Fig. 3).

### 3.2. Proton concentration and ionic strength effects on PC reduction

To study the deprotonation effect of the tyrosine side chain of the peptide, we measured $k_{int}$ under various pH conditions. The reciprocal initial rate constant increased linearly with the proton concentration (Fig. 4), and the reaction was not detectable at pH lower than 5.0. This relationship suggested that deprotonation of the peptide, presumably at the side chain of a Tyr, is important for the reaction, of which character was similar to that observed in the reduction of cyt *c* by TyrTyrPhe.

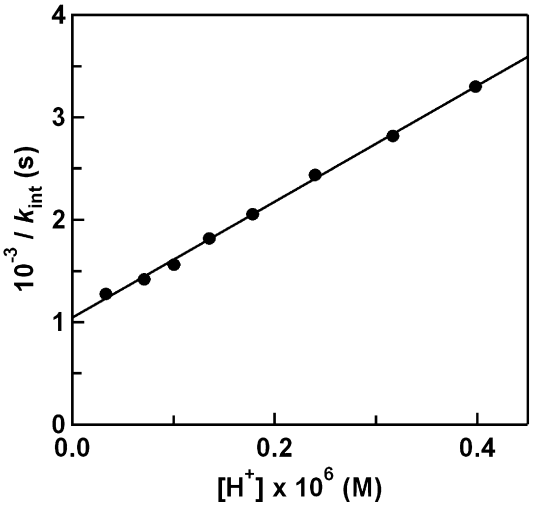


Fig. 4. Plots of $k_{int}$ vs [$H^+$], together with the least-squares-fitted line according to Eq. (5). Experimental conditions: PC, 50 μM; TyrTyrTyr, 1.0 mM; phosphate buffer (20 mM), 6.4–7.5; 15 °C.

Although $1/k_{int}$ increased linearly with [$H^+$] when 20 mM buffer was used (Fig. 4), $1/k_{int}$ did not increase linearly against [$H^+$] when 1 mM buffer was used. The non-linear character under the low buffer concentration may be due to the prominent effect of repulsion between negatively charged PC and deprotonated $(TyrTyrTyr)^-$, whereas the repulsion was suppressed under higher buffer concentration. These results suggest that the electrostatic interaction between PC and $(TyrTyrTyr)^-$ plays a significant role in the reaction.

To investigate in detail the electrostatic effect on the reaction between the negatively charged PC and the deprotonated peptide, $(TyrTyrTyr)^-$, $k_{int}$ was measured under different ionic strength [52]. The value of log $k_{int}$ increased linearly with the root of the ionic strength with a positive slope of 2.0 $M^{-1/2}$ (Fig. 5). The result shows that the interaction between PC and $(TyrTyrTyr)^-$ occurs during electron transfer.

### 3.3. Effect of LysLysLysLysTyrTyrTyr peptide on reaction of oxidized PC with TyrTyrTyr

We have previously shown that lysine oligopeptides interact with the negative patch of PC [32]. Therefore, we designed a tyrosine-containing peptide, which possessed a tetralysine site, so that the peptide could interact effectively with the negative patch of PC. The reduction rate of oxidized PC by TyrTyrTyr, as expected, was increased by an addition of the KKKKYYY peptide at pH 7.0 under air (Fig. 6). The reduction rate was increased much more by an addition of KKKKYYY instead of the same amount

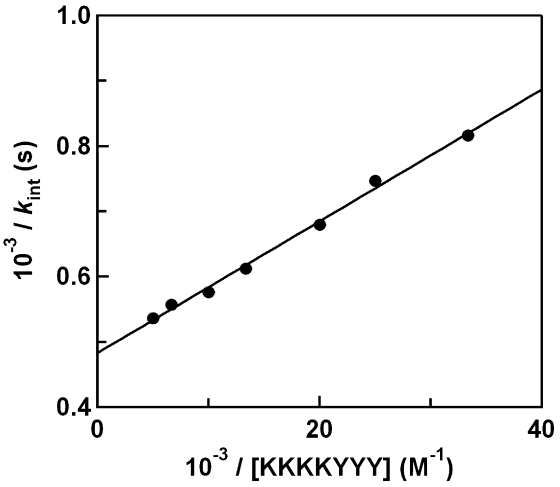


Fig. 6. Plots of $k_{int}$ vs KKKKYYY concentration in the presence of TyrTyrTyr. Experimental conditions: PC, 50 μM; TyrTyrTyr, 2.0 mM; KKKKYYY, 30–200 μM; phosphate buffer (1 mM), pH 7.0, 15 °C.

of tetralysine and TyrTyrTyr under the same condition. The reaction rate increased for about 80% by an addition of 100 μM KKKKYYY to the 50 μM PC–2 mM TyrTyrTyr solution, whereas it increased for about 35% by addition of 100 μM TyrTyrTyr and 100 μM KKKK to the same solution. PC, however, was not reduced at all by KKKKYYY without TyrTyrTyr under the same condition, as well as the DDDDYYY peptide could not reduce PC.

### 3.4. Mass spectra of reaction products

The reaction solution of PC with TyrTyrTyr was collected after 1 h of reaction, and the products were purified by the HPLC system. A small fraction with a retention time around 28.3 min was detected in the HPLC elution curve. This fraction corresponded to PC, since a similar fraction was detected in the HPLC elution curve of unreacted PC under the same condition. Another fraction eluted earlier and exhibited higher intensities at 280 nm than the PC fraction, and this higher-intensity fraction corresponded to the reaction products from TyrTyrTyr.

The ESI-LCMS spectrum of the peptide fraction showed molecular ion peaks at *m/z* 508.3, 530.4, 1015.7, and 1037.7 (Fig. 7). The ion peaks at *m/z* 508.3 and 530.4 were due to the protonated and $Na^+$ complex of TyrTyrTyr, whereas the ion peaks at *m/z* 1015.7 and 1037.7 corresponded well to the mass of the protonated and $Na^+$ complex of a dimer of TyrTyrTyr.

Since the tyrosyl radical is highly reactive towards phenols and usually produces a cross-linking at the *ortho* position of the phenol ring [6–10,17], the peptide must have been modified at the *ortho* position of a tyrosine side chain. Formation of a tyrosine dimer though a diphenol is consistent with the result obtained from the absorption spectrum, which exhibited a longer wavelength for the absorption of the reaction product than that of tyrosine (Fig. 2).

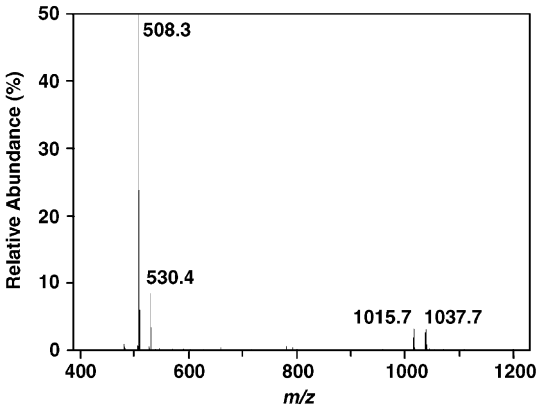


Fig. 7. ESI-LCMS spectrum of the reaction products. The mass spectrum was obtained for the products from the peptide after 1 h of reaction of PC with TyrTyrTyr.

Polymers, however, might have been produced from the tyrosyl radical, but no ion peak was detected above *m/z* 1200, probably due to production of various polymers with different molecular weights and/or increase in the hydrophobicity for the polymer. The radical products may also react with TyrTyrTyr and decrease the concentration of the unreacted TyrTyrTyr peptide, whereas they could also reduced and oxidized PC, respectively, which would make the reaction complicated.

## 4. Discussion

### 4.1. Mechanism of PC reduction by TyrTyrTyr

Oxidized PC was reduced by TyrTyrTyr at neutral pH (Fig. 2). Since the p$K_a$ values of the tyrosine side chains of TyrTyrTyr are 9.56, 9.95, and 10.92 [53], which are very close to the p$K_a$ value of the tyrosine side chain (p$K_a$ = 10.07), most of the phenol moieties of the TyrTyrTyr peptide should be protonated under the experimental conditions used in this study (pH 6.4–7.5). Deprotonation of the side chain of one of the tyrosines in the peptide, however, may occur for a small amount, although the p$K_a$ for the amino group of tyrosine is 9.11 and deprotonation of the amino group of the peptide occurs more easily. Since the phenolate species is necessary for the radical formation (see text below), we considered deprotonation at the tyrosine side chain. When the tyrosine side chain is deprotonated, the amino group should be protonated, due to the negative charge effect of the produced phenolate. The deprotonation process of the side chain of one of the tyrosines is described as below:

$$\mathrm{YYY} \overset{K_H}{\rightleftharpoons} (\mathrm{YYY})^- + \mathrm{H}^+ \quad (1)$$

where YYY and $(YYY)^-$ denote the protonated neutral and phenol-deprotonated negatively charged TyrTyrTyr peptides, respectively. Actually, 1 μM of deprotonated $(TyrTyrTyr)^-$ may exist in a 1 mM TyrTyrTyr solution at pH 7.0 for p$K_H$ = 10.

We can consider that reduction of PC with TyrTyrTyr occurs by interaction between PC and $(TyrTyrTyr)^-$, similarly as in the previous case for the reaction of cyt *c* with TyrTyrPhe [51]. We also assumed that electron transfer occurs reversibly, between oxidized PC and $(TyrTyrTyr)^-$ and between reduced PC and the produced $(TyrTyrTyr)^{\cdot}$ radical species. The radical can react further with unreacted peptides, and the scheme can be written as below:

$$PC_{ox} + (YYY)^- \overset{K_A}{\rightleftharpoons} PC_{ox}/(YYY)^- \underset{k_{-e}}{\overset{k_e}{\rightleftharpoons}} PC_{red}/(YYY)^{\cdot} \overset{k'}{\rightarrow} PC_{red}/(\text{reaction product}) \quad (2)$$

where $PC_{ox}$ and $PC_{red}$ represent oxidized and reduced PC, respectively. $K_A$ represents the association constant between oxidized PC and the deprotonated peptide, $k_e$ and $k_{-e}$ denote the electron transfer rate constants, and $k'$ denotes the rate constant for the reaction of the radical, respectively. The peptide with a radical is expressed as $(YYY)^{\cdot}$. If we write $K_E = k_e/k_{-e}$, we obtain

$$k_{int} = \frac{k'K_EK_A[(YYY)^-]}{1 + K_A[(YYY)^-]} \quad (3)$$

By considering Eq. (1), we obtain

$$\frac{1}{k_{int}} = \frac{K_H + [H^+]}{k'K_EK_AK_H}\frac{1}{[YYY]_T} + \frac{1}{k'K_E} \quad (4)$$

and

$$\frac{1}{k_{int}} = \frac{1}{k'K_EK_AK_H[YYY]_T}[H^+] + \frac{1 + K_A[YYY]_T}{k'K_EK_A[YYY]_T} \quad (5)$$

under the condition $[YYY] \gg [PC]$, where $[YYY]_T$ represents the total TyrTyrTyr concentration. The plot of $1/k_{int}$ vs $1/[YYY]_T$ exhibited a straight line with a slope of 1.2 M s (Fig. 3), whereas a linear increase of $1/k_{int}$ was observed as $[H^+]$ was increased (Fig. 4). These results substantiate the validity of the assumptions leading to Eqs. (4) and (5), which strongly support that the deprotonated species of the peptide reduces PC.

The value of $1/k_{int}$ was estimated to be very small for $1/[YYY]_T = 0$, which showed that $k'K_E$ was very large. Since $(YYY)^{\cdot}$ should be unstable and the equilibrium between $PC_{ox}/(YYY)^-$ and $PC_{red}/(YYY)^{\cdot}$ is usually shifted to the left as discussed below, $K_E$ should be small and thus the reaction of the radical should be fast. The $pK_a$ of the side chain and the voltage for oxidation of TyrTyrTyr do not differ significantly from those of a tyrosine monomer [51,53]. Since the tyrosyl radical reacts highly with a phenol [6], the produced radical should react effectively with a phenol of an unreacted TyrTyrTyr and thus PC would be kept reduced. We, however, could not detect a radical signal in the ESR spectrum of the reaction mixture, presumably due to the slow production and high reactivity of the tyrosyl radical.

TyrTyrTyr showed a higher PC reduction reactivity compared with tyrosine and TyrTyr. The higher reactivity for TyrTyrTyr may be due to the higher hydrophobicity of TyrTyrTyr than tyrosine and TyrTyr, where TyrTyrTyr peptides interacted with each other more effectively, although conformational changes in the peptides could also cause the difference in the reactivity. PC, however, was reduced much faster by TyrTyrLeu and TyrTyrPhe than by TyrTyr.

### 4.2. Interaction between PC and TyrTyrTyr

Both PC and the deprotonated $(TyrTyrTyr)^-$ peptide are negatively charged, and the electrostatic repulsion should prevent interaction between PC and TyrTyrTyr. Actually, $k_{int}$ increased with increasing the ionic strength (Fig. 5).

For a reaction between charged reactants with a same sign, the logarithms of the observed rate constants are expected to increase linearly against the root of the ionic strength ($I^{1/2}$) as shown in

$$\log k_{int} = \log k_0 + 1.02Z_AZ_B\sqrt{I} \quad (6)$$

where $Z_A$ and $Z_B$ represent the charges of the reactants and $k_0$ denotes the reaction rate constant at zero ionic strength, respectively. From the slope of this relationship for the reaction of PC and TyrTyrTyr (2.0 $M^{-1/2}$), the charge of the peptide interacting site for PC is estimated to be −2 by assuming the charge of $(TyrTyrTyr)^-$ as −1. The charge of the peptide interacting site is relatively small considering that there are many acidic residues at the negative patch of PC. The TyrTyrTyr peptide may interact with the PC hydrophobic patch, which is close to the active copper site and crucial for the PC–cyt *f* complex formation [30,44–47]. However, there is a certain repulsion effect. This effect was decreased by an increase in the ionic strength of the solution (Fig. 5). The reduction rate by TyrTyrTyr also increased a little when the charge of the negative patch of PC was reduced by mutation. A similar increase in electron transfer between PC and negatively charged $[Fe(CN)_6]^{4-}$ has been observed by decreasing the charge of the negative patch [54]. These results show that electrostatic interaction plays an important role in the reaction between PC and TyrTyrTyr.

### 4.3. Interaction between PC and KKKKYYY

We have previously shown that tetralysine interacts with the negative patch of PC [32]. By an addition of KKKKYYY to the PC–TyrTyrTyr solution, the reduction rate of PC was increased. The increase in the rate indicated that KKKKYYY interacted with PC effectively, and a similar equilibrium as in Eq. (6) may occur. The equilibrium can be written as

$$PC_{ox}/(KKKK(YYY)^-) \leftrightarrow PC_{red}/(KKKK(YYY)^{\cdot}) \quad (7)$$

where the side chain of the tyrosine next to the tetralysine site may form a radical, since it should be a little more deprotonated than other tyrosine side chains due to the electrostatic effect by the tetralysine site. KKKKYYY,

however, could not reduce PC without TyrTyrTyr. Although the tyrosyl radical, KKKK(YYY)$^{\bullet}$, may be produced by interaction of the peptide with PC, the radical may not be able to interact with an unreacted KKKKYYY. KKKKYYY is positively charged and it may not interact with each other due to electrostatic repulsion. As a result, back electron transfer from the reduced PC active site to the radical may occur. In the presence of both TyrTyrTyr and a small amount of KKKKYYY, PC was reduced much faster than by the same total peptide concentration with only TyrTyrTyr. KKKKYYY may interact with both PC and TyrTyrTyr like a mediator. KKKKYYY has a PC interacting site, KKKK, which would make the peptide interact with PC more effectively through the negative patch, and the produced KKKK(YYY)$^{\bullet}$ radical would interact with TyrTyrTyr by hydrophobic interaction, whereas TyrTyrTyr may also react directly with the hydrophobic patch of PC. These results support that hydrophobic interaction between the tyrosine-containing peptides is important for the reaction.

### 4.4. Reaction product from TyrTyrTyr

Since the additional molecular ion peak detected in the mass spectrum of the products from the reaction of PC with TyrTyrTyr corresponded well with the mass of a tyrosine dimer, a tyrosyl radical should have generated during the reaction. The gradual decrease in the reduction rate of PC with time should be due to a large decrease in the TyrTyrTyr concentration, where the decrease in the concentration may be caused by reaction of the radical product with an unreacted TyrTyrTyr. The tyrosyl radical could react with an unreacted TyrTyrTyr presumably at the side chain of a tyrosine in the peptide, and the product radical may also react further with another tyrosine as a chain reaction. This radical chain reaction may cause a decrease in the peptide concentration faster than the reduction of PC. The product dimer tyrosine may also be reactive and reduced PC, and the reaction would become complicated. No significant difference in the reaction rate, however, was observed between the reaction under nitrogen and oxygen atmospheres, which showed that reduction of PC with TyrTyrTyr is independent with molecular oxygen. This result supports that a radical coupling between the peptides occurs during the reaction, and similar characters were observed for the reaction between cyt *c* and TyrTyrPhe [51].

## 5. Conclusion

Oxidized PC was reduced at neutral pH with the TyrTyrTyr oligopeptide, whereas it was not reduced effectively with a monomeric tyrosine or TyrTyr under the same condition. PC interacted with deprotonated $(TyrTyrTyr)^{-}$ for the reaction. Although KKKKYYY did not reduce PC in the absence of TyrTyrTyr, the reduction rate of PC with TyrTyrTyr increased by an addition of KKKKYYY, which possessed a PC interacting site. By the mass analysis, a TyrTyrTyr dimer peptide was suggested as a reaction product, which indicated that tyrosyl radicals were formed during the reaction.

We assumed that electron transfer occurs reversibly between PC and tyrosine-containing peptides to produce $PC_{ox}/(TyrTyrTyr)^{-} \leftrightarrows PC_{red}/(TyrTyrTyr)^{\bullet}$ or $PC_{ox}/(KKKK(YYY)^{-}) \leftrightarrows PC_{red}/(KKKK(YYY)^{\bullet})$ equilibriums. Reaction of the tyrosyl radical with the unreacted TyrTyrTyr peptides enhances reduction of PC by preventing back electron transfer from the reduced copper of PC to the tyrosyl radical of the peptide. This study shows that a metalloprotein could be reduced with a tyrosine-containing oligopeptide.

## 6. Abbreviations

| | |
|---|---|
| PC | plastocyanin |
| cyt *f* | cytochrome *f* |
| PSI | photosystem I |
| cyt *c* | cytochrome *c* |
| TFA | trifluoroacetic acid |
| $k_{int}$ | initial rate constant |
| LCMS | liquid chromatography–mass spectrometry |
| ESI | electrospray ionization |
| $PC_{ox}$ | oxidized plastocyanin |
| $PC_{red}$ | reduced plastocyanin |
| $k'$ | rate constant for the reaction of the radical |
| $I$ | ionic strength |
| $k_0$ | rate constant at zero ionic strength |

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