Plastocyanin−Peptide Interactions. Effects of Lysine Peptides on Protein Structure and Electron-Transfer Character

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Received March 3, 1998

Abstract: Structural change of plastocyanin (PC) due to the interaction with lysine peptides (Lysptd's) has been studied by absorption, resonance Raman, and electrochemical measurements and by measuring the electron transfer between PC and cytochrome *c* (cyt *c*) in the presence of Lysptd. Absorption spectral changes which were observed when Lysptd's up to penta-lysine were added to PC solution have been ascribed by resonance Raman studies to the change in the active site Cu−cysteine geometry upon binding of Lysptd to the PC negative patch. The same spectral changes were observed for the PC−cyt *c* interaction. Electrochemical measurements showed that the redox potential of PC increases upon Lysptd binding, suggesting that Lysptd's induce a structural change in PC through the copper ligating cysteine residue to make the copper site adapted for facile electron transfer. Lysptd's competitively inhibited the electron transfer from reduced cyt *c* to oxidized PC, which indicated that they function as models of the PC interacting site of proteins. The effects of Lysptd on electron transfer are explained as competitive inhibition due to neutralization of the PC negative patch by formation of PC·Lysptd complexes. The electron-transfer rate from reduced cyt *c* to oxidized PC and the inhibiting effect of Lysptd decreased upon decreasing the net charge of the negative patch by mutation. The structural change of PC was also found to decrease significantly with these mutants. The present observations strongly support that the PC negative patch is the dominant cyt *c*/*f* molecular recognition site and open up the possibility that charged peptides can be used for studying protein−protein interactions in a systematic way.

Introduction

Plastocyanin (PC), a mobile electron-transfer protein existing in the thylakoid lumen of photosynthetic organisms, is reduced by cytochrome *f* (cyt *f*) and oxidized by the reaction center chlorophyll ($P700^+$) in the photosystem I (PSI) complex.[1−3] 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.[4−7] This protein is classified as a Type 1 copper protein from its spectroscopic and electronic properties, exhibiting a low-energy ligand-to-metal charge-transfer (LMCT) band near 600 nm in its absorption spectrum and having a relatively high redox potential.[8,9] Although the structure−function relationship is one of the major topics in protein researches and it is of fundamental importance to know the structural change upon protein−protein complex formation, to our knowledge, there is no detailed study on the molecular interaction induced structural changes of PC.

PC, cyt *c*, and cyt *f* have many charged residues on their surfaces. It is well established that PC and cyt *f* or cyt *c* recognize each other through electrostatic interactions, and X-ray crystallographic studies of cyt *c* showed that basic amino acids are distributed around its heme edge.[10−14] A recent X-ray structural determination of chloroplast cyt *f* revealed that a lysine (Lys) residue-rich positive patch exists at a solvent-exposed site, which has been predicted as the docking site for PC.[15] On the other hand, on the basis of the crystal structures of oxidized and reduced plant PC's,[4−7] two highly conserved sites of PC have been considered as molecular recognition sites for its redox partners, cyt *f*, cyt *c*, and PSI: One site is located at the Cu-coordinating, solvent-accessible histidine (Cu-adjacent hydrophobic patch), and the other site is located at another solvent-accessible site containing acidic residues near a tyrosine residue

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S0002-7863(98)00711-2 CCC: $15.00
Published on Web 07/31/1998

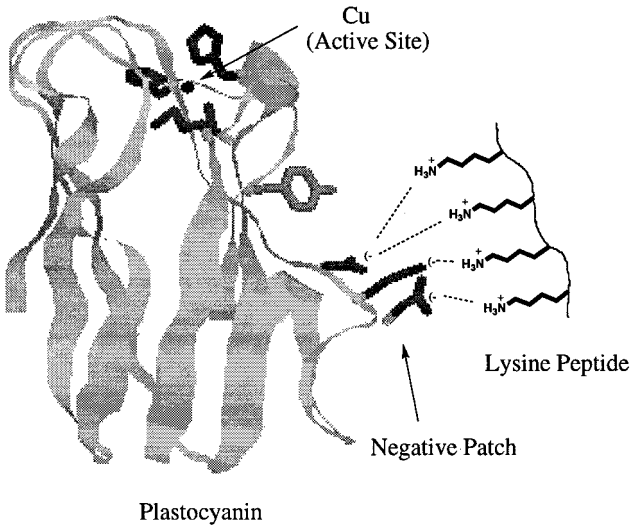

Figure 1. Schematic view of the interaction between PC and tetra-Lys. Note that poplar PC is depicted but silene and spinach PC have four consecutive residues at their negative patch.

(Cu-remote negative patch) (Figure 1). Early studies on electron transfer from reduced PC to $[Co(phen)_3]^{3+}$ suggested that both are molecular recognition sites for cyt *f*,[16,17] but computational analyses based on electrostatic forces[18,19] and other experimental studies[20−28] have indicated that the Cu-remote negative patch is the cyt *c*/*f* molecular recognition site, while the electron transfer is believed to occur through the hydrophobic patch of PC.[19] Recently, Kostić et al. proposed that PC and cyt *c* or cyt *f* bind and react with each other in different configurations resulting from the protein−protein interaction termed as the gating process for electron transfer,[29−32] showing possible configurations for the diprotein complex by computer simulation.[19,33]

The constants for association between PC and cyt *f* or cyt *c* have been obtained by measuring the increase of the Soret band intensity of cyt *f* or cyt *c* on PC binding.[34−36] Investigations on the formation of the PC−cyt *c* complex by NMR techniques using the $[Cr(NH_3)_6]^{3+}$ complex have been attempted, but existence of a number of binding sites for $[Cr(NH_3)_6]^{3+}$ made the discussion complicated.[37,38] A recent NMR study on the Cd-substituted PC−cyt *c* complex indicated that the PC−cyt *c* complex consists of a highly dynamic ensemble of structures.[39] Tyrosine (Tyr) 83 which is located between the negative patch and the copper active site has been proposed to be involved in the electron transfer, since the reaction rate decreased upon replacement of Tyr with leucine.[36] Ullmann and Kostić recently demonstrated by molecular dynamics a possibility of a cation-π interaction at the Tyr-83 site, which might be important for the electron transfer.[19]

Table 1. Amino Acid Sequences of the Negative Patch for Wild-Type and Mutant PC's

species	amino acid sequence[a]	net charge of the negative patch
wild-type	-Asp-Glu-Asp-Glu-	−4
M1	-Asn-Glu-Asp-Glu-	−3
M2	-Asp-Lys-Asp-Glu-	−2
M3	-Asn-Lys-Asp-Glu-	−1
M4	-Asp-Lys-Lys-Glu-	0

[a] Underlines indicate modified amino acid residues.

Positively charged lysine peptides (Lysptd's), which may correspond with the cyt *c*/*f* amino acid residues exposed to the solvent, may likewise interact with the consecutive aspartic acid (Asp) and glutamic acid (Glu) residues of the negative patch of PC (Figure 1). As a matter of fact, charged peptides inhibited the electron transfer from reduced cyt *c* to oxidized PC.[40] To gain insights into the structural changes upon protein−protein interactions and the structure−function relationship, we performed spectroscopic and electrochemical investigations of the effects of Lysptd on the PC structure and function by using negative patch mutants of PC and Lysptd's as models for the PC interacting site of proteins. From the present observations on the PC−Lysptd interactions, we propose that electrically charged peptides may be useful for structural studies on other electrostatic protein−protein interactions.

Experimental Section

Preparation of Samples. *Silene pratensis* (white campion) wild-type and negative patch mutant PC's (M1−M4, Table 1) were expressed in *Escherichia coli* and purified by published methods.[20,41] Absorption and EPR spectra of site-directed mutant PC's were the same as those of wild-type PC, indicating that mutations of amino acid residues at the negative patch do not affect the Cu active site in solution.[20] Moreover, the X-ray crystallographic structure of M4 mutant PC showed good correspondence with that of wild-type PC.[42] The purity of the

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enzymes were confirmed by the absorbance (Abs) ratio between at 280 and 597 nm ($Abs_{280}/Abs_{597} < 1.35$).

PC was dissolved in 10 mM phosphate buffer, pH 7.4, or 10 mM Tris-HCl buffer, pH 7.3, with a certain amount of Lysptd. Tetra-Gly and Lysptd's (= Lys, di-Lys, tri-Lys, tetra-Lys, and penta-Lys) (Gly = glycine and Lys = lysine) were purchased from Sigma. Bovine heart cyt *c* purchased from Sigma was purified with a CM52 (Whatman) column after reducing it by adding ascorbic acid dissolved in buffer. Oligopeptides were first dissolved in 10 mM phosphate buffer, pH 7.4, or 10 mM Tris-HCl buffer, pH 7.3, with a peptide concentration of 40 mM, and then the pH values and concentrations of the peptide solutions were readjusted to pH 7.4 (or 7.3) and 10 mM, respectively, by using 10 mM phosphate (pH 7.4) or Tris-HCl (pH 7.3) buffer and 0.1 M NaOH dissolved in 10 mM phosphate or Tris buffer. The concentrations of PC and cyt *c* were adjusted by their absorption spectra.

Spectroscopic Measurements. Absorption spectra of PC in the presence of Lysptd's were measured at 15 °C on a Shimadzu UV 3101PC spectrophotometer.

Resonance Raman Measurements. Resonance Raman scattering was excited at 591 nm with an Ar^+ ion laser (Spectra Physics, 2017)-pumped dye laser (Spectra Physics, 375B) with Rhodamine 6G and detected with a triple polychromator (JASCO, NR-1800) equipped with a CCD detector (Princeton Instruments). The slit width and slit height were set to be 100 μm and 15 mm, respectively. The excitation laser beam power (at the sample point) was adjusted to 60 mW. Measurements were carried out at room temperature with a spinning cell (3000 rpm). The data accumulation time was 400 s. Raman shifts were calibrated with CCl_4, and the accuracy of the peak positions of the Raman bands was ± 1 cm^{-1}.

Electrochemical Measurements. Cyclic voltammetry was carried out at room temperature with a voltammetric analyzer (Bioanalytical Systems, 100B). A 2-(diethylamino)ethanethiol (Sigma)-modified gold electrode, prepared as described previously,[43] was used as a working electrode, and a gold wire and an Ag/AgCl electrode were used as counter and reference electrodes, respectively.[43,44] The midpoint redox potentials were calibrated by using $[Co(phen)_3]^{2+/3+}$.[1]

Kinetic Measurements. The electron-transfer rate constants from reduced cyt *c* to oxidized PC in the presence of Lysptd's were obtained by monitoring the absorbance at 420 nm with an Otsuka Denshi RA601 stopped-flow equipment attached with an oscilloscope. A 10 μM solution of PC in 10 mM Tris-HCl buffer, pH 7.3, containing 10 mM NaCl was mixed at 15 °C with 1 μM cyt *c* in the same buffer. The inhibitory effect of charged peptides on the electron-transfer rate was studied with a PC solution containing Lysptd (0−600 μM), the peptide concentration being 0−300 μM after mixing PC and cyt *c* solutions. Although the rate constants were very sensitive to the experimental conditions, especially the buffer and salt concentrations, the relative rate values were reproducible.

Results

Oxidized PC−Lysine Peptide Interactions. (1) Absorption Spectral Studies. Protonation of Lysptd is reasonably presumed at pH 7.3−7.4, since the pK_a value for the ϵ-amino group of Lys is 10.71 at 25 °C ($I = 0.1-0.2$).[45] The absorption spectra of oxidized PC with and without penta-Lys and their difference spectrum are shown in Figure 2A. A factor of 0.98−1.02 was used to make the peak and trough areas equal, which differences demonstrate the absorbance changes. Peaks (~620 and ~460 nm) and troughs (~540 and ~700 nm) were detected in the difference absorption spectrum, and their positions were different from the peak position (597 nm) of oxidized PC, which has been assigned to the cysteine thiolate (S_{cys})-to-Cu(II) charge-transfer band.[8,9] The PC−Lysptd interaction caused the 597-nm band to shift a little to a longer wavelength and increase the intensity of the 460-nm band. The difference absorption spectra for PC with and without various Lysptd's shown in Figure 2B exhibit the peaks for penta- and tetra-Lys but no significant peak for tri-Lys, di-Lys, and Lys. The difference absorption spectra for wild-type and negative patch mutant PC's with and without tetra-Lys (Figure 2C) show that the changes at around 600 nm due to addition of Lysptd are hardly detectable for mutant PC's.

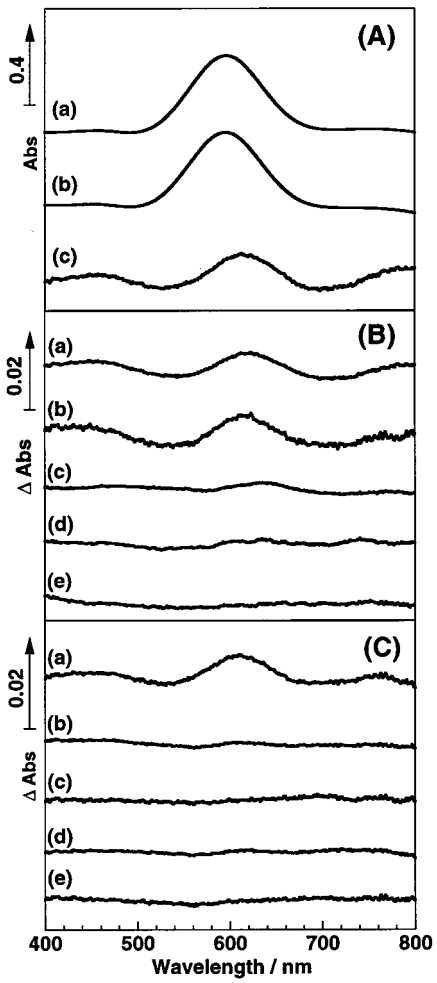

Figure 2. (A) Absorption spectra of (a) PC (100 μM) with penta-Lys (330 μM) and (b) PC without it and (c) their difference spectrum (a − b) multiplied by 20. (B) Difference absorption spectra between PC (100 μM) with and without various Lysptd's (Lys−penta-Lys). The concentrations of Lysptd's were (a) penta-Lys, 260 μM; (b) tetra-Lys, 360 μM; (c) tri-Lys, 530 μM; (d) di-Lys, 1.2 mM; (e) Lys, 2.4 mM. (C) Difference absorption spectra of wild type and negative patch mutant PC's (100 μM) with and without tetra-Lys (360 μM): (a) wild type, (b) M1, (c) M2, (d) M3, and (e) M4. Phosphate buffer (10 mM), pH 7.4, was used.

(2) Resonance Raman Studies. Figure 3A exhibits the resonance Raman (RR) spectra in the 200−600 cm^{-1} region for PC (500 μM) with and without penta-Lys (660 μM) excited

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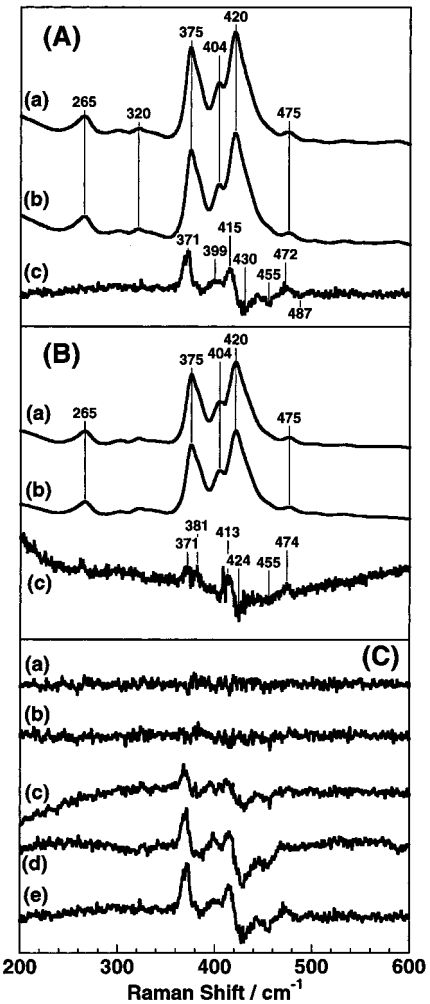

Figure 3. (A) RR spectra in the 600−200 cm^{-1} region for (a) PC (500 μM) with penta-Lys (660 μM) and (b) PC without it and (c) their difference spectrum (a − b) multiplied by 10. The ordinate scales in spectra a and b are normalized with the intensity of the 265 cm^{-1} band. (B) RR spectra in the 600−200 cm^{-1} region for (a) PC (500 μM) with cyt *c* (500 μM) and (b) PC without it and (c) their difference spectrum multiplied by 15. The RR spectrum of cyt *c* was also subtracted in spectrum c. (C) Difference RR spectra between PC (500 μM) and PC with various Lysptd's: (a) Lys, 6.0 mM; (b) di-Lys, 3.0 mM; (c) tri-Lys, 1.3 mM; (d) tetra-Lys, 900 μM; (e) penta-Lys, 660 μM. Experimental conditions: slit height, 15 mm; slight width, 100 μm; excitation wavelength, 591.0 nm; laser power, 60 mW. Phosphate buffer (10 mM), pH 7.4, was used.

at 591.0 nm and their difference spectrum, where peaks were detected at 371, 399, 415, and 472 cm^{-1}, while troughs were detected at 430, 455, and 487 cm^{-1}. Several bands at 374−475 cm^{-1}, which are due to the coupling of the Cu−S stretch with $S_\gamma-C_\beta-C_\alpha-N$ deformation modes of the coordinated cysteine residue,[46−48] were affected by added Lysptd, showing that the cysteine geometry was perturbed by Lysptd.

The difference RR spectrum in the 200−600 cm^{-1} region between PC (500 μM) with and without cyt *c* (500 μM) excited at 591.0 nm is shown in Figure 3B. Comparison of parts A and B of Figure 3 clearly indicates that the difference pattern obtained for PC with and without penta-Lys is similar to that obtained between PC with and without cyt *c*. Structural changes of cyt *c* have been detected by RR in the cyt *c*−cytochrome *c* oxidase system.[49,50] The difference RR spectra between PC with and without Lysptd's with various lengths show that the intensities of the peaks are larger for penta- or tetra-Lys than for tri-Lys and hardly detectable for di-Lys or Lys (Figure 3C).

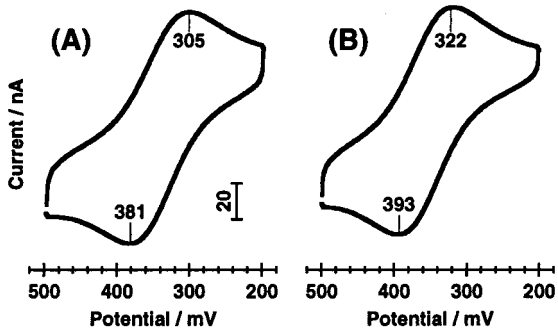

Figure 4. Cyclic voltammetry of (A) PC (100 μM) and (B) PC with penta-Lys (260 μM). Phosphate buffer (10 mM), pH 7.4, was used.

Table 2. Midpoint Redox Potentials ($E_{midpoint}$) of PC (100 μM) in the Presence of Lysptd's (Lys−Penta-Lys) (vs NHE)[a]

Lysptd[b]	$E_{midpoint}$/mV	Lysptd[b]	$E_{midpoint}$/mV
—	343	tri-Lys	349
Lys	344	tetra-Lys	358
di-Lys	345	penta-Lys	358

[a] Measured in 10 mM phosphate buffer, pH 7.4, at 15 °C; error ±5 mV. [b] The concentrations of Lysptd's were as follows: penta-Lys, 260 μM; tetra-Lys, 360 μM; tri-Lys, 530 μM; di-Lys, 1.2 mM; and Lys, 2.4 mM.

(3) Electrochemical Studies. Cyclic voltammograms obtained for PC in the presence of a certain amount of various Lysptd's showed well-defined quasi-reversible faradaic responses (Figure 4), with midpoint potentials listed in Table 2. Lys-containing peptide-modified electrodes have been used for measurement of redox potentials.[51] The peak separation decreased in the presence of penta- or tetra-Lys compared to those in the presence of tri-Lys, di-Lys, or Lys, and the midpoint potentials of PC were about 10−15 mV higher in the presence of penta- or tetra-Lys.

Electron Transfer between PC and Cyt *c*. To investigate the nature of the interactions between PC and Lysptd's in more detail, we performed stopped-flow measurements on the electron transfer between reduced cyt *c* and oxidized PC. Figure 5 shows that the inverse plots of the observed electron-transfer rate constants (k_{obs}) versus the initial concentrations of Lysptd's (0−300 μM) give a straight line. The inhibition of the electron-transfer became prominent as the length and concentration of Lysptd increased, but it did not change when a same amount of tetra-Gly was added (data not shown), showing that the effect of the peptide terminal charges of the $-COO^-$ and NH_3^+ groups can be neglected.

Figure 6 summarizes the observed electron-transfer rate constants between reduced cyt *c* and oxidized wild-type and mutant PC's in the presence of 0, 90, or 150 μM tetra-Lys. The rate constants for mutant PC's also decreased as the concentra-

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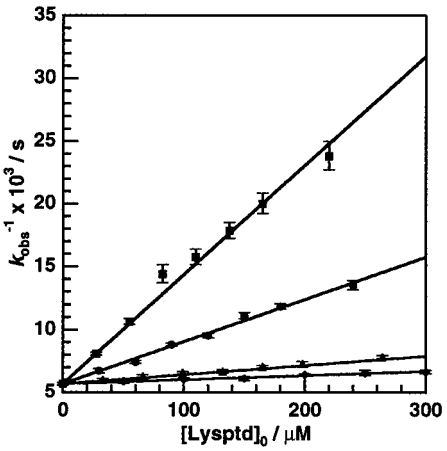

Figure 5. Plots of the reciprocal electron-transfer rate constants ($1/k_{obs}$) for the oxidized PC-reduced cyt *c* system against the initial concentrations (0−300 μM) of Lysptd's (di- (◆), tri- (▲), tetra- (●), and penta- (■) Lys), together with least-squares fitted lines according to eq 3. Tris buffer (10 mM), pH 7.3, containing 10 mM NaCl was used.

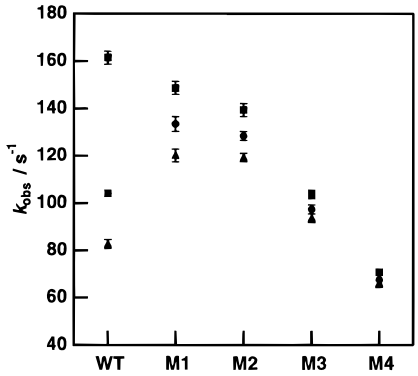

Figure 6. Plots of the electron-transfer rate constants (k_{obs}) for the oxidized wild-type and mutant PC-reduced cyt *c* system against the initial concentrations of tetra-Lys (0 (■), 90 (●), 150 (▲) μM). Tris buffer (10 mM), pH 7.3, containing 10 mM NaCl was used.

tion of Lysptd increased. As the net charge of the negative patch of PC decreased (Table 1), the cyt *c*−PC electron-transfer rate constant decreased. The effects of Lysptd's and mutant PC's on the electron-transfer rate revealed that Lysptd's having positive charges interact with the negative patch of PC and thus decrease the rate.

Discussion

Oxidized PC−Lysine Peptide Interaction at the PC Negative Patch. Despite the importance of investigations on the protein−protein interaction, it is difficult to deduce the resulting structural change of one protein against the other for the PC−cyt *c*/*f* system by the absorption spectrum because these proteins show overlapped absorption bands in the visible region. Since Lysptd's served as competitive inhibitors of the electron transfer from reduced cyt *c* to oxidized PC, we propose that Lysptd's function as models for the PC interacting site of proteins. The difference RR spectrum obtained for PC with and without penta-Lys was similar to that for PC with and without cyt *c* (Figure 3A,B), supporting that Lysptd's are good models for cyt *c*.

Lysptd's caused changes in the absorption spectrum of PC at around 600 nm (Figure 2), while tetra-Gly and NaCl did not (data not shown). Since this band is assigned to the S_{cys}-to-Cu(II) LMCT,[8,9] the structure of the Cu site (at least the Cu−S_{cys} bond) should be altered by addition of these Lysptd's. This structural change should be based on an equilibrium between PC and Lysptd because the intensities of the peaks and troughs increased as the concentration of penta-Lys increased (data not shown). On the other hand, lack of the spectral change upon addition of tri-Lys, di-Lys, or Lys suggests that the PC−Lysptd association constants for shorter peptides are small and that PC effectively recognizes penta- or tetra-Lys. The decrease in the peak separation of the cyclic voltammogram of PC in the presence of penta- or tetra-Lys compared to that in the presence of tri-Lys, di-Lys, or Lys, and without Lysptd also supports that only penta- and tetra-Lys interact with PC effectively. The observed change in the absorption spectrum disappeared when the NaCl concentration was increased to 100 mM (data not shown), which supports that the structural change is induced when Lysptd electrostatically interacts with the PC negative patch. The spectral change dependent on the net charge of the negative patch in Figure 2C suggests that these mutant PC's do not associate with Lysptd's due to the decrease of the negative charge at the negative patch. This structural change of PC could be induced by the charge−π interaction recently proposed by Ullmann and Kostić.[19]

Structural Change at the Cu Site Induced by Lysine Peptide. RR spectroscopy is a powerful method for investigating the character of the Cu−S_{cys} bond in Type 1 copper proteins by exciting the Raman scattering near the S_{cys}-to-Cu(II) charge-transfer band.[46−48] When tetra- or penta-Lys was added to the PC solution (Figure 3C), the bands at 375−475 cm^{-1} slightly shifted to lower frequencies, and small increase of the intensities of some lower frequency bands in this region was observed. Since the weighted average frequency based on the S-isotope dependence has a correlation with the Cu−S_{cys} distance,[52] the results indicate that the Cu−cysteine bond was weakened a little upon addition of Lysptd. Intensity increase of the 460-nm absorption band has been explained by the structural change from a trigonal planar toward a more tetrahedral Cu site geometry causing lengthening of the Cu−S_{cys} bond in Type 1 copper proteins.[53] The RR results correspond with the increase of the 460-nm absorption band in the difference absorption spectrum (Figure 2), supporting that the Cu−S_{cys} bond length for PC bound with Lysptd is longer compared to that without Lysptd. This structural change could be regarded as a result of the gating process as reported by Kostić et al.[29−32] The intensities of the peaks seen in the RR difference spectrum due to the PC−Lysptd interaction were smaller for tri-Lys and not detectable for di-Lys or Lys. According to the absorption difference spectra discussed above, tetra- and penta-Lys interact with the negative patch of PC while tri-, di-Lys, and Lys do not, so that the structural changes at the Cu site should have been induced by PC−Lysptd interaction at the PC negative patch.

EPR measurements of the copper atom of PC gave no significant change upon addition of tetra- or penta-Lys. This could be because EPR is more sensitive to the Cu−N bond geometry, while the structural change seen from the 375−475 cm^{-1} bands in the RR spectra are due to coupling of the Cu−S stretch with $S_\gamma-C_\beta-C_\alpha-N$ deformation modes of the coordi-

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nated cysteine residue.[46−48] In line with this, the 265 cm^{-1} band, which is assigned to the Cu−His stretching,[54] did not show any change.

On the other hand, the midpoint potential of PC increased in the presence of tetra- or penta-Lys compared to that in the presence of tri-Lys, di-Lys, Lys, or without Lysptd (Table 2), while the midpoint potentials of mutant PC's did not change in the presence of the same amount of tetra- or penta-Lys (data not shown). Therefore, we infer that the above-mentioned structural change raises the redox potential of Cu and makes it easier for PC to receive an electron from its redox partner.

Kinetic Consideration on PC−Lysine Peptide Binding and Its Effect on Electron Transfer. It is of fundamental importance to know how proteins recognize their electron accepting and/or donating partners, and there have been a number of studies on the electron transfer between proteins.[55−58] Electron transfers between PC and cyt *f* or cyt *c* have been studied extensively,[20,23−28,31,32,35,59,60] where the positively charged cyt *c* has been used as a model for cyt *f*. Redox reactions between PC and small molecules have also been investigated extensively, and Sykes et al. have previously discovered in an elegant way that small inorganic compounds can inhibit the electron transfer between PC and cyt *c* or cyt *f*.[17,21,61−63]

In this study, the electron transfer rate constant decreased as the net charge at the negative patch decreased for site-directed mutants of PC, especially for M4 mutant PC (Figure 6). Since the geometry of amino acid residues of M4 mutant PC is not modified significantly from wild-type PC,[42] the decrease in the Lysptd's inhibitory effect is due to the decrease of the net charge of the negative patch. This result indicates that the inhibition is attributed to the electrostatic interaction of the peptides, which interferes with the formation of a PC·cyt *c* complex.

The observed inhibition may be interpreted by considering formation of two complexes: a PC·cyt *c* complex, where electron transfer occurs subsequently, and a PC·Lysptd complex, which competitively inhibits the formation of the PC·cyt *c* complex and thus inhibits the electron transfer. The complex formations are expressed by the following equations:

$$PC_{ox} + \text{Lysptd} \overset{K_i}{\rightleftharpoons} (PC_{ox}\cdot\text{Lysptd})_R \quad (1)$$

$$PC_{ox} + \text{cyt } c_{red} \overset{K_{OS}}{\rightleftharpoons} (PC_{ox}\cdot\text{cyt } c_{red})_R \xrightarrow{k_e} (PC_{red}\cdot\text{cyt } c_{ox})_R \quad (2)$$

where K_i and K_{OS} are the association constants for PC_{ox}·Lysptd and PC_{ox}·cyt c_{red} complexes, respectively, and k_e represents the electron-transfer rate constant. The suffixes ox and red refer to the oxidized and reduced states, respectively, and R denotes that the complex is formed at the Cu-remote negative patch of PC. If we write the observed rate constant as k_{obs}, $K_{OS}k_e$ as k, and the initial concentrations of PC and Lysptd as $[PC]_0$ and $[\text{Lysptd}]_0$, respectively, we obtain the relationship

$$\frac{1}{k_{obs}} = \frac{K_i}{k[PC]_0}[\text{Lysptd}]_0 + \frac{1}{k[PC]_0} \quad (3)$$

Plots of $1/k_{obs}$ against $[\text{Lysptd}]_0$ gave lines shown in Figure 5, substantiating the validity of the assumptions leading to eq 3. k (=(35.2 ± 0.4) × 10^6 M^{-1} s^{-1}) and K_i values (Table 3) were obtained from least-squares fitting with eq 3. Since the fitting was successful, it is reasonable to assume that only one Lysptd is bound to the negative patch of PC.

Table 3. Association Constants (K_i) for PC−Lysptd Complexes[a]

length of Lysptd	K_i/M^{-1}	length of Lysptd	K_i/M^{-1}
di	550 ± 130	tetra	5900 ± 300
tri	1400 ± 200	penta	15300 ± 1000

[a] In 10 mM Tris-HCl buffer, pH 7.3, containing 10 mM NaCl.

Table 4. Association Constants (K_i) for Mutant PC−Tetra-Lys Complexes[a]

species	K_i/M^{-1}	species	K_i/M^{-1}
M1	1420 ± 160	M3	730 ± 40
M2	1040 ± 110	M4	490 ± 40

[a] In 10 mM Tris-HCl buffer, pH 7.3, containing 10 mM NaCl.

The association constant between a 5+ redox inactive complex and reduced PC is reported to be larger than that between a 4+ redox inactive complex and reduced PC, while the constants for the oxidized PC and same redox inactive compounds were in the opposite order.[17,62,63] This discrepancy could be caused by the differences in the size and shape of the complexes. In the present systems, K_i became larger as the charge of the Lysptd, i.e., the number of lysyl residues, increased (Table 3). The larger K_i values for PC with tetra- and penta-Lys than with tri-, di-Lys, and Lys may suggest that PC effectively recognizes sites with more than three positive charges compared to those with three or less charges, which would enable PC to bind its redox partner more selectively.

The electron-transfer inhibition by Lysptd decreased significantly as the net charge of the negative patch of PC decreased. Association constants, K_i, for mutant PC−tetra-Lys complexes were calculated by using eq 3 and the values in Figure 6 and are listed in Table 4. The K_i values decreased as the net charge of the negative patch decreased (wild-type > M1 > M2 > M3 > M4), also supporting that the negative patch is the Lysptd interacting site. The inhibitory effect of tetra-Lys decreased considerably when the net negative charge of the PC negative patch was two or less, indicating the importance of having more than three negative charges at the negative patch for the interaction with tetra-Lys.

Conclusion

Absorption spectral studies of wild-type and negative patch mutant PC's with various lengths of Lysptd showed that PC interacts with Lysptd at its negative patch, and it interacts effectively only with Lysptd equal to or longer than tetra-Lys. RR spectral changes of PC were the same for PC-cyt *c* and PC−Lysptd interactions, indicating that Lysptd's are good models for the PC recognition site of proteins. Interaction of PC with tetra- or penta-Lys gave rise to a certain structural change around the copper active site, as seen from the spectral changes detectable in the absorption and RR spectra. RR measurements suggested that the interaction of PC with tetra- or penta-Lys at its PC negative patch caused the $Cu-S_{cys}$ bond to become longer, and electrochemical studies showed this

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structural change raised the redox potential of Cu, making PC fitted for receiving an electron from its redox partner.

Lysptd's such as tetra- and penta-Lys have been found to function as competitive inhibitors for the electron transfer from reduced cyt *c* to oxidized PC, which is explained by neutralization of the PC negative patch by formation of PC·Lysptd complexes. The reduced cyt *c* to oxidized PC electron-transfer rate constant decreased with the net charge of the negative patch of site-directed mutant PC's, indicating that the electron transfer is affected by the net charge of the PC negative patch. The decrease in the electron transfer rate constant shows that the negative patch is the dominant pathway. Interestingly, the association constants for the PC−Lysptd complexes reveal that PC effectively recognizes the sites with four or more than four positive charges.

Taken together, these observations indicate that Lysptd's, especially tetra- and penta-Lys, function as models for the PC interacting site of proteins.

Acknowledgment. The authors thank Dr. Tsuyoshi Inoue and Prof. Yasushi Kai of Osaka University for information of the silene wild-type and mutant PC's before publication. This work was supported by a Grant-in-Aid for Scientific Research (No. 09304062) from the Ministry of Education, Science, Sports, and Culture of Japan, for which we express our thanks.

JA980711L