Reduction and Oxidation Processes of Blue Copper Proteins, Azurin, Pseudoazurin, Umecyanin, Stellacyanin, Plantacyanin, and Plastocyanin Approached by Cyclic and Potential Step Voltammetries

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Direct electrochemistry of a series of blue copper proteins: azurin, pseudoazurin, umecyanin, stellacyanin, plantacyanin, and plastocyanin has been performed at a gold electrode modified with di-4-pyridyl disulfide and/or at a bare glassy carbon electrode. Well-resolved cyclic voltammograms with peak separation, $\Delta E_p = 55$—100 mV were obtained, allowing the facile determination of the redox potential of blue copper proteins. Protein molecules associate with the electrode surface through both electrostatic and hydrophobic interactions, of which the predominant one differs according to the combination of protein and electrode. Double-step voltammetry showed that redox processes of blue copper proteins depend profoundly on the translocation of the molecules (diffusion and/or change of orientation) at the electrode surface. The heterogeneous rate constants at pH 6.0 and 25 °C for both reduction and oxidation processes were independently determined to be the order of 10^{-3} to 10^{-4} cm s^{-1} by single-step voltammetry and were compared with those determined by cyclic voltammetry. Further, activation parameters for redox processes of some blue copper proteins have been determined from temperature dependence studies. The reaction pathway of blue copper proteins was discussed.

Blue copper proteins are relatively small proteins with a molecular weight of 10000—20000.[1] The biological roles of blue copper proteins include, together with iron-sulfur proteins and cytochromes, electron transfer in respiration and photosynthesis.

Crystal structures of plastocyanin,[2] azurin,[3] pseudoazurin,[4] plantacyanin,[5] and amicyanin[6] have been determined. All of them have the analogous features in their structure and active site. A copper ion is placed at the "north" side of the protein molecules, being coordinated by 1Cys, 2His and 1Met or 1Gln.[7] The imidazolyl group of one of His ligands, beneath which the copper ion is positioned, is surrounded by hydrophobic residues. Since this imidazole edge is exposed to solvent, it has been believed to be the intrinsic electron-transfer pathway. However, in the case of plastocyanin, Tyr83 has also been supposed to serve as another pathway for electron transfer, since it is adjacent to one of ligands, Cys84 and is also to the "eastern" acidic patch, the docking site with the cyt bf complex.[8]

Together with structural studies, a number of kinetic studies using stopped-flow, flash-photolysis, and pulse-radiolysis[9] have been performed for native proteins, chemically modified proteins with a redox active center such as Ru complex, and mutated proteins. Although the electrochemical approach is advantageous in that both reduction and oxidation reactions can be performed under the same experimental condition by changing the applied voltage on the working electrode, its application to blue copper proteins

has been limited to plastocyanin[10] and azurin.[11] However, we have recently performed direct electrochemistry of three basic proteins, stellacyanin, plantacyanin, and pseudoazurin at an amorphous glassy carbon electrode in the absence of mediator and promoter.[12,13] In this paper, we show the direct electrochemistry of six blue copper proteins: azurin, pseudoazurin, umecyanin, plantacyanin, stellacyanin, and plastocyanin at a di-4-pyridyl disulfide-modified Au electrode and at a bare amorphous glassy carbon electrode. The manner of the interaction between these proteins and electrodes has been investigated by cyclic voltammetry and double-step voltammetry. The rate of reduction and oxidation has been independently determined by single-step voltammetry. Activation parameters have also been determined for some systems.

Experimental

Proteins and Materials. *Pseudomonas aeruginosa* azurin and horseradish umecyanin were obtained from Sigma and were purified by using Sephadex G-75. Pseudoazurin was isolated from *Achromobacter cycloclastes* as reported.[14] Stellacyanin was purified from Chinese lacquer (*Rhus vernicifera*) latex obtained from Takano and Co., Kanazawa.[15] Plantacyanin and plastocyanin were isolated from cucumber peel according to the literature.[16] Purity of these blue copper proteins was checked from A_{280}/A_{600} ratio and HPLC. Reduced proteins were prepared by voltammetry or by treating resting proteins with a tiny amount of L-ascorbate or dithionite, followed by dialysis against buffer under N_2. Proteins were never fatally denatured as were evidenced from absorption spectra before

and after electrochemical measurements. Phosphate buffer (pH 6.0, 0.1 M) was used throughout measurements unless specially noted ($M = mol\,dm^{-3}$).

Apparatus and Procedures.　The cell system is composed of a Ag/AgCl reference electrode, a Pt plate counter electrode and a glassy carbon working electrode or a Au electrode modified with di-4-pyridyl disulfide according to literature.[17] Measurements were performed by dipping the cell in a thermostatted bath under N_2. Prior to each experiment the electrode was polished with 0.3 μm alumina/water slurry and sonicated briefly, followed by rinsing with water. Voltammetries have been usually performed in phosphate buffer (pH 6.0, 0.1 M) at 25 °C except that for plastocyanin in 0.05 M acetate buffer containing 0.02 M NaCl and 0.1 M $MgCl_2$ at 3 °C.

Cyclic voltammetry was performed on a Yanagimoto P-1100 Voltammetric Analyzer. The scan rate was 1 to 1000 $mV\,s^{-1}$. Single- and double-potential step voltammetries were carried out using a Fuso HECS972 potential sweep unit attached to a home-built timer unit and a Fuso HECS-980 potentiostat as reported in our previous paper.[13] The current was preamplified by using a Fuso 972-2 head box. The switching time was changed over 1 to 999 ms. The data were stored in a transient converter, a Riken Denshi model TECD, and were recorded on an X–Y recorder.

Results

Cyclic Voltammetry.　Cyclic voltammetry has been performed for all six proteins by using a bare glassy carbon electrode and a Au electrode modified with di-4-pyridyl disulfide (Fig. 1). Well-resolved voltammograms were obtained except for plastocyanin and azurin at the bare glassy carbon electrode. The glassy carbon electrode we used were amor-

phous, differing from the previous studies on plastocyanin[10] and azurin[11] at the edge-oriented pyrolytic graphite.

Peak separations between anodic and cathodic peaks (ΔE_p) were 54 to ca. 100 mV, indicating that the electron transfer between blue copper proteins and electrodes is quite rapid in spite of the voltammetry of macromolecules. The current ratios of cathodic and anodic peak currents, i_a/i_c did not deviate much from 1 except in the case of plastocyanin. The cyclic voltammetry for plastocyanin was highly dependent on pH. The voltammogram was almost symmetric at pH 5, but the anodic current was larger than the cathodic current at more acidic pH and the cathodic current was, in turn, larger than the anodic current at higher pH. Nevertheless, all these findings suggest that direct electrochemistry of six blue copper proteins is feasible at a Au electrode modified with di-4-pyridyl sulfide and at a glassy carbon electrode (amorphous or edge-oriented). The midpoint potential vs. pH plots for plastocyanin and azurin (data not shown) were sigmoidal, in accordance with those determined by potentiometric titration studies.[18]

Buffer Concentration Dependence of Cyclic Voltammetry.　In order to reveal the manner of interaction between protein molecule and electrode, buffer concentration dependence was investigated for all systems. The electrochemistry of stellacyanin at the glassy carbon electrode was almost independent of buffer concentration as reported in our previous paper.[13] On the other hand, when the modified Au electrode was used, the cyclic voltammogram with nar-
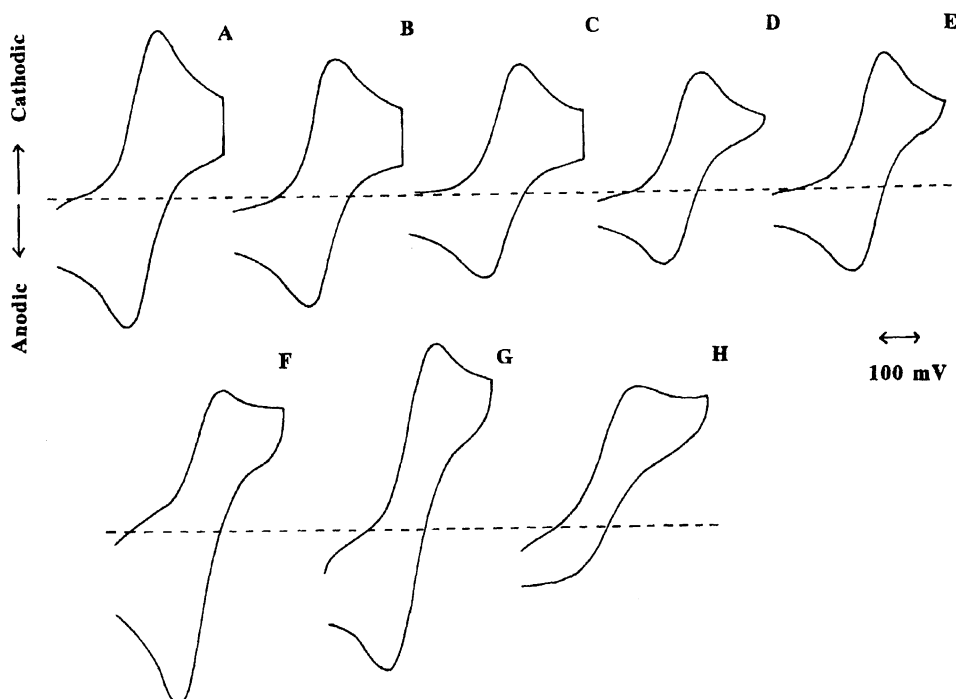

Fig. 1.　Cyclic voltammograms of pseudoazurin at a modified Au electrode (A), azurin at a modified Au electrode (B), stellacyanin at a modified Au electrode (C), plantacyanin at a modified Au electrode (D), umecyanin at a glassy carbon electrode (E), and plastocyanin at a modified Au electrode (F at pH 4.5, G at pH 5.0, H at pH 6.5). Measurement conditions: protein concentration ca. 100 mM except plastocyanin (41 μM), phosphate buffer except plastocyanin (50 mM acetate buffer containing 20 mM NaCl and 100 mM $MgCl_2$), pH 6.0, sweep rate 1—5 $mV\,s^{-1}$, temp 25 °C except plastocyanin (3 °C), reference Ag/AgCl).

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rower peak separation was obtained in 0.005 M phosphate buffer than in 0.2 M phosphate buffer, although the midpoint potential was kept constant (data not shown).

Cyclic voltammograms of pseudoazurin at the modified Au electrode gave narrow peak separation, $\Delta E_p = 54$ to 60 mV, although the midpoint potential increased with decreasing buffer concentration (Table 1). At the carbon electrode the midpoint potential decreased with increasing buffer concentration, while the peak separation increased with increasing buffer concentration.
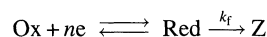
Plantacyanin, azurin, and umecyanin showed the similar behavior. The midpoint potential slightly increased with decreasing buffer concentration, although peak separation did not change regardless of buffer concentration.

Plastocyanin at the modified Au electrode gave redox waves in the presence of relatively highly concentrated electrolytes such as NaCl and MgCl$_2$, contrary to the studies using the edge-oriented pyrolytic graphite.[10,11] The protein structure might have been deformed at low electrolyte concentration because of too strong interaction between pyridyl residues of the promoter and the acidic patch at the eastern side of the protein molecule. However, plastocyanin had not

Table 1. Effect of Phosphate Buffer Concentration for the Cyclic Voltammetry of Blue Copper Proteins

Protein (Electrode)	Buffer/M	$E°$/mV	ΔE_p/mV
Stellacyanin (C)	0.001	189	95
	0.03	179	88
	0.1	181	82
	0.4	180	85
Pseudoazurin (Au)	0.001	330	56
	0.005	333	58
	0.02	318	56
	0.1	287	54
	0.2	275	60
(C)	0.005	263	59
	0.05	244	98
	0.1	250	79
	0.2	231	132
Plantacyanin (Au)	0.02	331	70
	0.1	321	70
	0.2	311	70
(C)	0.02	333	78
	0.1	318	89
	0.2	313	87
Azurin (Au)	0.005	349	55
	0.03	349	68
	0.1	332	65
	0.2	328	66
Umecyanin (Au)	0.005	330	58
	0.02	317	65
	0.1	289	80
	0.2	274	60
(C)	0.005	322	55
	0.02	314	60
	0.1	294	80
	0.2	277	55

Protein Concentration, ca. 100 μM; Temp, 25 °C; pH 6.0; Sweep rate, 1—5 mV s^{-1}.

been denatured as was ascertained by the absorption spectrum after measurements.

Sweep Rate Dependence. All figures showing the current against square root of sweep rate (not shown) were linear at least unto ca. 200 mV s^{-1} for all systems, insuring that the reaction processes were diffusion-controlled, although the linearity for plastocyanin was maintained only at the slower sweep rate.

Double-Potential Step Voltammetry. In order to compare the rates of the electron transfer for reduction and oxidation processes and to obtain information on how protein molecules behave at the electrode surface, double-potential step voltammetry has been performed. An example of how to get a set of data for plantacyanin at the carbon electrode is shown in Fig. 2. The anodic and cathodic currents, which were obtained by subtracting control data, were determined at times t and $(t-t_f)$ (i.e. at the same time after switching) and their ratio i_a/i_c was plotted against the switching time in Fig. 3 (The results at the carbon or the modified Au electrode were shown for each blue copper protein). The current during the second potential step depends on the relative amount of the reduced species diffusing to the electrode and away from it. The value 0.29 is expected for the completely reversible process ($k_f = 0$).[19]

$$Ox + ne \rightleftharpoons Red \xrightarrow{k_f} Z$$

Umecyanin and pseudoazurin gave value ca. 0.3 regardless of switching time. Plantacyanin and azurin showed values less than 0.3 at short switching times, while the i_a/i_c
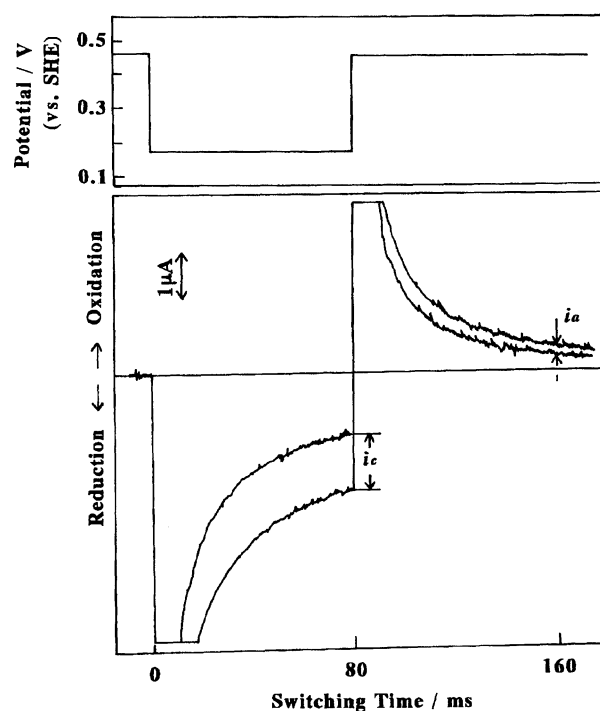

Fig. 2. Double-potential step voltammetry of plantacyanin at a di-4-pyridyl disulfide-modified Au electrode (protein concentration, 130 μM; 0.1 M phosphate buffer; pH 6.0, switching time 80 ms).
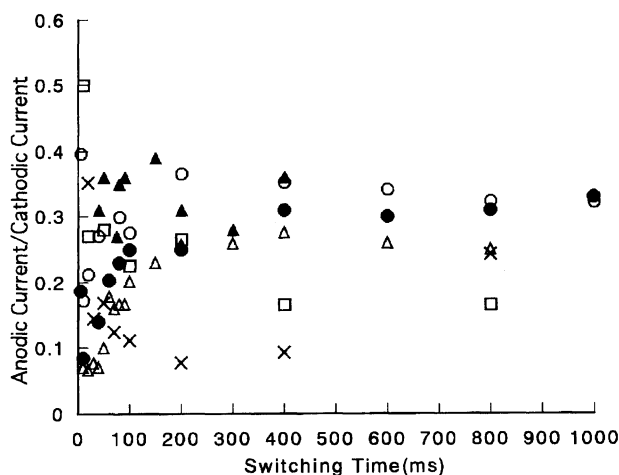
Fig. 3. Ratios of anodic and cathodic currents in double-step voltammograms for plastocyanin (□) (10 μM in 50 mM acetate buffer (pH 6.0) containing 20 mM NaCl and 100 mM MgCl₂ at 3 °C), azurin (△) (77 μM in 0.2 M phosphate buffer (pH 6.0) at 25 °C), pseudoazurin (○) (119 μM in 0.005 M phosphate buffer (pH 6.0) at 25 °C), plantacyanin (●) 130 μM in 0.1 M phosphate buffer (pH 6.0) at 25 °C), umecyanin (▲) (100 μM in 0.2 M phosphate buffer (pH 6.0) at 25 °C, and stellacyanin (×) (120 μM in 0.033 M phosphate buffer (pH 6.0) at 25 °C. The data for pseudoazurin and stellacyanin were obtained by using a glassy carbon electrode and the data for other proteins by using a Au electrode modified with di-4-pyridyl disulfide.

value increased with increasing switching time, reaching 0.3 at ca. 200 ms. In the case of stellacyanin at the glassy carbon electrode, i_a/i_c value was ca. 0.35 at 20 ms, followed by the decrease with increasing switching time and the successive increase to give a trough at ca. 200 ms. However, at the modified Au electrode the behavior was similar to those of plantacyanin and azurin, although the i_a/i_c value did not reach ca. 0.3 within 1 s. In the case of plastocyanin, the i_a/i_c value continued to decrease within the experimental limit of 1 s, because a portion of the reduced plastocyanin was strongly adsorbed and denatured at the electrode surface.

Single-Potential Step Voltammetry. Single-potential step voltammetry was performed in order to independently determine the rate constants for both the reduction and oxidation processes. It was assumed that the simple charge transfers

$$Ox + e \longrightarrow Red,$$
$$Red + e \longrightarrow Ox$$
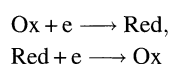
take place at short time after stepping voltage, usually 12.5 ms. The current i at a constant potential is related to the diffusion limited current, i_d by the equation:

$$i/i_d = \pi^{1/2}\lambda \exp \lambda^2 \operatorname{erfc}\lambda.$$

This is applicable for a planar electrode (semi-infinite linear diffusion). The kinetic parameter λ is given by

$$\lambda = kt^{1/2}D^{-1/2},$$

where D is the diffusion constant and t the sampling time.[13,19] The Cottorell constant, i_d was obtained from a polarization curve such as Fig. 4 for umecyanin at 25 °C and 12.5 ms. The diffusion constant was calculated from the Cottorell equation

$$i_d = nFD^{1/2}c(\pi t)^{-1/2},$$

where n, F, and c represent number of electrons passed per act of the overall reaction (here 1), Faraday constant, and concentration of protein in bulk of solution, respectively.[13,19]

The heterogeneous rate constant at $E°$ was tabulated in Table 2 together with that determined according to Nicholson's method using the peak separation in cyclic voltammogram.[20] Almost all of the reduction and oxidation for each protein proceeded at the order of 10^{-3} cm s^{-1}.

Activation Parameters for Reduction and Oxidation Processes. Single-step voltammetry has been performed at several temperatures in order to determine the activation parameters, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. The typical Eyring plots to obtain the activation parameters are shown in Fig. 5 and the results are summarized in Table 3. Since the experiment for plastocyanin can be performed only at a low temperature near 0 °C, the activation parameters could not be obtained. The activation parameters for stellacyanin were excluded from Table 3, since reproducibility was not very good.

Discussion

Of the twelve systems for combinations of six blue copper proteins and two kinds of electrodes, ten systems (not plastocyanin or azurin at the amorphous carbon electrode) were suitable for direct electrochemistry. The facile direct electrochemistry might be realized because the intrinsic biological role of blue copper protein is electron transfer. In line with this, the reason why direct electrochemistry of cytochrome
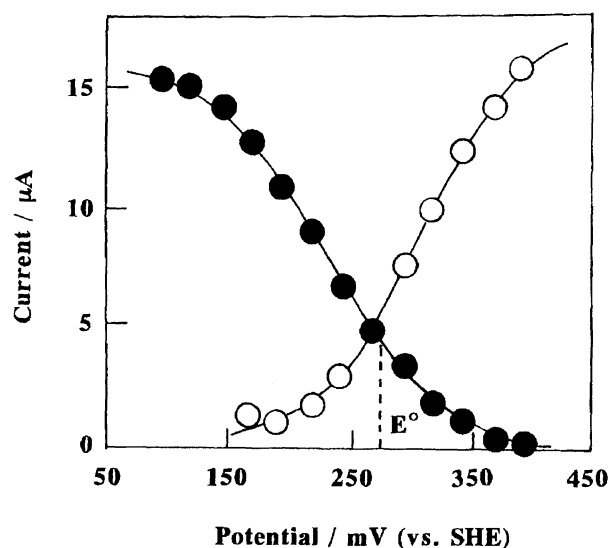

Fig. 4. Polarization curves for the reduction (●) and the oxidation (○) of umecyanin (114 μM, 25 °C, 0.2 M phosphate buffer, pH 6.0) obtained from single-step voltammetry at a glassy carbon electrode. The switching time was 12.5 ms.

Table 2.   Heterogeneous Electron-Transfer Rate Constant of Blue Copper Proteins at an Au Electrode Modified with Di-4-pyridyl Disulfide or at a Glassy Carbon Electrode (25 °C, 0.1 M buffer)

Protein (Electrode)	$10^3 k/\mathrm{cms}^{-1}$ from CV[a]	$10^3 k/\mathrm{cms}^{-1}$ from Single-step voltammetry	
		Reduction	Oxidation
Plastocyanin (Au)	0.044(3 °C)	0.85	
Azurin (Au)	3.3	1.3	1.4
Pseudoazurin (Au)	>20	14	7.6
(C)	>22	3.1	1.8
Plantacyanin (Au)	4.8	2.6	2.4
(C)	2.5	(0.4)	(1.1)
Stellacyanin (Au)	1.3	0.36	
(C)	3.3	1.4	1.1
Umecyanin (Au)	2.0	2.6	
(C)	4.6	10	14

a) Determined from peak separation according to the Nicholson's method by using the equations $i_p=0.4463\times10^{-3}n^{3/2}F^{3/2}A(RT)^{-1/2}c^*~v^{1/2}$ and $\psi=(D_O/D_R)^{\alpha/2}k_OD_O\pi(nF/RT)^{1/2}$ where i_p, n, F, A, R, T, c^*, v, ψ, D_O, D_R, α, and k_O denote peak current, number of electrons passes per act (here 1), Faraday constant, area of electrode, gas constant, temperature (here 25 °C), concentration in bulk of solution, sweep rate, rate parameter in cyclic voltammetry, diffusion coefficient of the oxidized species, diffusion coefficient of the reduced species, transfer coefficient, and standard heterogeneous rate constant of the oxidized species, respectively.
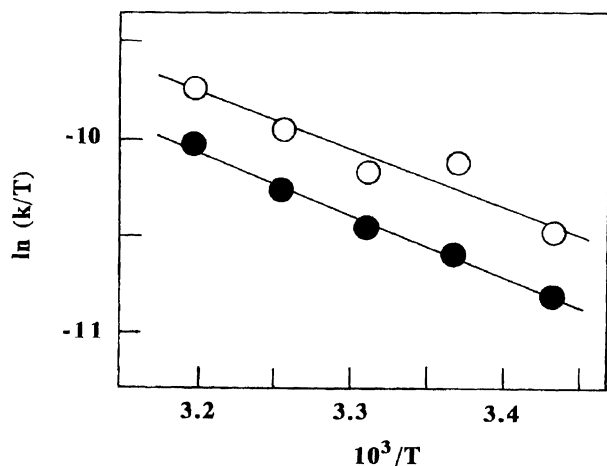

Fig. 5.   Eyring plots for the reduction (●) and the oxidation (○) of umecyanin (114 μM, 25 °C, 0.2 M phosphate buffer, pH 6.0) obtained from single-step voltammetry using a glassy carbon electrode at 12.5 ms.

c[21] has been widely studied will be that this protein is specially designed as to be suitable for electron transfer. Since it has been reported that plastocyanin[10] and azurin[11] give the redox waves at an edge-oriented pyrolytic graphite, di-

rect electrochemistry of all six blue copper proteins appears to be possible by using an amorphous and/or edge-oriented pyrolytic graphite electrode and the modified Au electrode.

Both of the anodic and cathodic currents were similar for all systems except plastocyanin. As shown in Fig. 1 (F, G, H), the feature of voltammograms of plastocyanin was highly dependent on pH. At pH 5 the voltammogram was almost symmetric, but at higher pH's the cathodic current was larger than the anodic current and at lower pH's the anodic current was larger than the cathodic current. This might have occurred because plastocyanin is the only blue copper protein having the dual electron-transfer pathway (the northern route involving the ligand His87 and the eastern route involving Tyr83).[8] According to the X-ray crystal structure of reduced plastocyanin at pH ca. 4, the copper (I) ion is dissociated from the His87 imidazole to give the redox inactive form.[22] However, this seems to contradict the fact that the anodic current was apparently larger than the cathodic current at low pH's (see Fig. 1). On the other hand, the facile autoreduction of plastocyanin at an alkaline pH[23] is in accordance with the fact that the cathodic current increased with increasing pH. Modified proteins such as ruthenated azurines[24] have contributed to the understanding of the biological electron

Table 3.   Activation Parameters for Reduction and Oxidation Processes Determined by Single-Step Voltammetry

Protein	(Electrode)	Reduction		Oxidation	
		$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$
		kJ mol^{-1}	J K^{-1} mol^{-1}	kJ mol^{-1}	J K^{-1} mol^{-1}
Plantacyanin	(Au)	28	−202	15	−241
	(C)	16	−237	28	−196
Umecyanin	(C)	27	−194	23	−203
Pseudoazurin	(Au)	13	−239	15	−240
	(C)	6.5	−87		
Azurin	(Au)	52	−127	63	−91

transfer, exhibiting that even the artifact electron transfer becomes possible whenever a sufficient driving force, the redox potential difference between redox centers, is applied. However, it will be quite natural that electron transfer to and from the Cu center proceeded only via the His87 imidazolyl group[8] in the present electrochemical processes.

The buffer concentration dependency in Table 1 gives information about interactions between protein and electrode surface. The surface of the carbon electrode is hydrophobic, while the oxidized carbon groups are partly formed by abrasing, as shown by XPS.[21] On the other hand, the pyridyl residue at the Au electrode surface is not charged under the experimental conditions. Accordingly, the hydrophobic interaction should mainly operate in the case of the carbon electrode, and the hydrophilic interaction (hydrogen bond and dipolar interaction) in the case of the modified Au electrode. In order to realize the electrochemical communication between electrodes and blue copper proteins, the access of the macromolecules with an appropriate orientation is a prerequisite. Therefore, the proper selection of the pertinent electrolyte and its concentration is particularly important to promote well-resolved redox waves.

Redox behavior of stellacyanin at the carbon electrode was almost independent of buffer concentration, indicating that both the hydrophobic and hydrophilic interactions operate in the access of the protein molecule towards the electrode surface.[13] Although stellacyanin is a highly basic protein (the isoelectric point, pI=9.9)[15] and accordingly, its surface is highly positively charged, hydrophobic residues encircling the ligand His92 assist the access of the north pole towards the electrode surface.[7]

Pseudoazurin (pI=8.4) gave well-resolved redox waves with narrow ΔE_p over a wide range of buffer concentration regardless of the type of the electrode used. However, the redox potential increased with decreasing buffer concentration at both Au and C electrodes. Pseudoazurin has several Lys residues encircling the northern His area, Lys38, Lys46, Lys57, and Lys77 (adjacent to Cys78)[25] and accordingly, the neutralization of these residues might be indispensable to avoid denaturation of the protein molecule at the electrode surface.

In analogy with this, plantacyanin (pI=10.6),[26] azurin (pI=5.4),[27] and umecyanin (pI=5.8)[28] gave the formal potentials identical to those obtained by potentiometric titration. However, with decreasing buffer concentration, redox potential increased because of the possible denaturation. Otherwise, redox potential of these proteins might virtually shift in diluted buffer, although no data is available.

Double-potential step voltammetry has never been applied to a macromolecular system before we have recently applied it to stellacyanin at a glassy carbon electrode.[13] The i_a/i_c value seemed to continue to decrease with increasing switching time at < 20 ms,[19] because of translocations (diffusion and/or change of the orientation at the electrode surface) of the protein molecule. However, it began to approach the value 0.3 with increasing switching time, giving a trough at switching time of ca. 200 ms. Thus, it appears that dou-

ble-step voltammetry is an effective technique to display the behavior of a protein molecule at an electrode surface. However, when the modified Au electrode was used, the i_a/i_c value did not return to 0.3 within 1 s. Translocations of the stellacyanin molecule might have been hampered, since it contains 50% of its weight as sugar (three Asn residues carry carbohydrate chains and may interfere with the proper association of its northern area with the electrode surface).[15]

Umecyanin and pseudoazurin gave ca. 0.3 as the i_a/i_c value regardless of the switching time (Fig. 2), indicating that the protein molecules translocated to take the redox active orientation promptly within 1 ms. On the other hand, plantacyanin and azurin showed less than 0.3 values even at the short switching time, 5 ms, but the i_a/i_c value increased continuously with increasing switching time, finally reaching 0.3 at ca. 200 ms. This seems to suggest that translocations of plantacyanin and azurin are not as rapid as in the cases of umecyanin and pseudoazurin, while they are not as slow as in the case of stellacyanin. Plastocyanin showed different behavior: i_a/i_c value was 0.5 at 10 ms and ca. 0.3 at 20 to 50 ms, but it gradually continued to decrease further with increasing switching time. This suggests that the electron shuttling took place between the protein molecule whose orientation was fixed and the modified Au electrode at a switching time shorter than 10 ms. However, translocations of the protein molecule took place with increasing switching time and further, a portion of the reduced protein might begin to be denatured at the electrode surface at > 100 ms.

Single-step voltammetry was performed in order to independently determine the heterogeneous rate constant for reduction and oxidation. The results in Table 2 indicated that electron transfers proceed at the order of 10^{-3} cm s^{-1} in most cases. The rate is as rapid as to those of the electron transfers of small redox molecules. The reason why such a prompt electric communication was realized at interfaces between blue copper proteins and electrode surfaces will be that the biological role of blue copper proteins is intrinsically electron transfer. Direct electrochemistry of proteins whose biological role is not electron transfer, for example that of myoglobin, has become possible only recently.[29] The rate constants for the oxidation of plastocyanin and stellacyanin were not obtained because the reduced protein molecules might deform at the electrode surface and sufficient currents were not obtained. Nevertheless, both the reduction and the oxidation proceeded at comparable rates for all blue copper proteins (Table 2), suggesting that electron transfers are performed utilizing the same pathway, the northern His residue.[8] If all blue copper proteins have two electron transfer pathways as plastocyanin does, the rates of the reduction and oxidation might differ more prominently. Although another route via the uncoordinated His has been supposed in the case of azurin,[9] the recent mutation studies excluded such the possibility[30] Electron transfers from the remote cystine[31] and ruthenated redox center[24] have been performed, affording basic information about driving force, electron transfer pathway, and reorganization accompanying redox state change. However, most of these electron transfers have been

performed via artifact mechanisms. On the other hand, the present electric communication between blue copper proteins and the carbon or the modified Au electrode dose not seem to be performed utilizing nonbiological pathways. The rate of oxidation for plastocyanin could not be obtained because of the possible denaturation of the reduced protein at the electrode surface. The heterogeneous rate constant of the quasireversible system is usually determined from peak-to-peak separation in the cyclic voltammogram according to Nicholson's method,[20] giving the averaged electron-transfer rate of reduction and oxidation. However, since ΔE_p was less than 59 mV in some cases of pseudoazurin, the lower limit was indicated (Table 2) to avoid overstimation. The slight discrepancy between the heterogeneous rate constant estimated from cyclic voltammetry and potential-step chronoamperometry might be caused by the possible occasional adsorption of blue copper proteins on the electrode surface prior to the electron transfer and/or some limit to applying the equations to the irreversible process.

Further, we determined activation parameters from temperature dependence studies (Fig. 5 and Table 3). Activation enthalpies were relatively small as observed in many processes with electron-transferring proteins.[9] This is presumably because the reorganization energy is small in the biological electron transfers. On the other hand, activation entropies have quite large negative values. This shows that the protein molecules should be highly directionally oriented at the electrode surface just before the electron transfer occurs. Activation enthalpy and activation entropy for both reduction and oxidation processes did not differ much for all blue copper proteins, suggesting again that electron transfer might be performed via the same pathway for both reduction and oxidation.

Figure 6 shows how the electron transfer might become possible at the interface between the northern His ligand in blue copper proteins and the pyridyl promoter on the modified electrode surface. In the case of carbon electrode, the oxygenated groups are supposed to be the active site.[21] Therefore, the His imidazolyl group and the oxygenated groups at the carbon electrode or the pyridine moiety at the Au electrode surface interact to realized the electron transfer through the bridge. The only exception might be plastocyanin having the two electron-transfer pathways.[8] The eastern pathway might be possible if the acidic patch interacts properly with pyridyl moieties. In order to get information about this, we are now studying using various promoters.[32]

Concluding Remarks

Cyclic voltammetry of six blue copper proteins could be realized by using a di-4-pyridyl disulfide-modified Au electrode and an amorphous glassy carbon electrode except for plastocyanin and azurin. Both the hydrophobic and electrophilic interactions operate for the proper access of the protein molecules towards the electrode surfaces. Double-step voltammetry showed that the northern His ligand is the electron-transfer pathway both in the reduction and oxidation processes. The translocation of the active site (the rotation of protein molecules on the electrode surface and/or the diffusion) appears to occur at less than 1 ms to ca. 200 ms. The heterogeneous electron-transfer rate and activation parameters for reduction and oxidation processes were independently determined, suggesting again the reduction and oxidation are performed via the coordinating imidazolyl group.
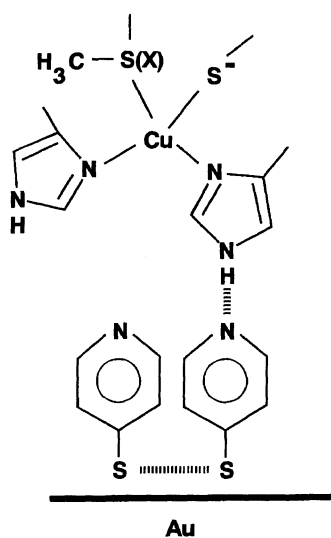
Fig. 6. A schematic drawing to show the possible interaction of a blue copper protein and the modified Au electrode surface to realize the direct-electrochemistry.

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