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Direct Electrochemistry of the Blue Copper Proteins Pseudoazurin, Plantacyanin, and Stellacyanin

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Direct electrochemistry of three blue copper proteins, *Achromobacter cycloclastes* IAM 1013 pseudoazurin, *Cucumis sativus* plantacyanin, and *Rhus vernicifera* stellacyanin, is achieved at a glassy-carbon electrode over the pH range 5–11 in the absence of mediators and promoters. The formal potentials $E^{\circ\prime}$ for pseudoazurin, plantacyanin, and stellacyanin are 0.28, 0.32, and 0.18 V (vs NHE), respectively, at pH 6.0. These values are identical with those previously determined by the conventional potentiometric titrations. The three blue copper proteins behave as effectively symmetrical redox systems ($i_{pa} \sim i_{pc}$) over the pH range 5–11. However, cathodic and anodic wave peak potentials of the three blue copper proteins show diverse pH dependencies, suggesting that an electron might be passed to and from the copper center via different pathways. The oxidation–reduction processes are almost reversible or quasi-reversible at pH <10, as indicated by the fairly narrow cathodic and anodic peak separations (ΔE_p) of 60–90 mV). At pH >10, ΔE_p values begin to increase because of the protein structure change, which is partly reflected in the absorption and ESR spectra.

Introduction

Electron transfer is one of vital chemical processes of organisms. Some metalloproteins such as cytochromes, iron–sulfur proteins, and blue copper proteins are of concern in photosynthesis, respiration, etc.[1] The goal of this study is not only elucidating how the electron-transfer chains are constituted by relevant proteins but also elucidating how the redox couples recognize each other and how the intermolecular and intramolecular electron transfers occur.

Blue copper proteins contain one blue copper ion (type 1 copper) in the relatively small protein molecules: they fall in the molecular weight range 10000–20000, the larger one possessing an appreciable amount of carbohydrate.[2] Of the blue copper proteins, plastocyanin, which is involved in photosynthesis of higher plants and green alga,[3] and azurin, which is involved in the respiration process of a variety of bacteria,[4] have received special attention and have been well characterized.[5] Compared to plastocyanin and azurin, many other blue copper proteins have been less studied.[6]

In the present communication we show the direct (unmediated) electrochemistry of three blue copper proteins, *Achromobacter cycloclastes* IAM 1013 pseudoazurin, *Cucumis sativus* plantacyanin, and *Rhus vernicifera* stellacyanin at a glassy-carbon electrode. Although the electrochemistry of plastocyanin[7] and rusticyanin[8] has been successfully performed, addition of a mediator or inorganic cation such as Mg^{2+} or the use of a modified glassy-carbon electrode, which is capable of interacting reversibly and specifically with a protein molecule, has been indispensable in order to promote redox waves. As far as we know, this is the first report of the direct electrochemistry of blue copper proteins at an untreated glassy-carbon electrode in the absence of a mediator, modifier, promoter, or additive (inorganic salt etc., except potassium phosphate as buffer). The formal potentials, $E^{\circ\prime}$, were identical with those determined by the conventional potentiometric method,[9] and accordingly, cyclic voltammetry is an extremely convenient technique for determining the redox potential of some blue copper proteins. The observed diverse pH dependencies suggested that the coming and going of electrons between the glassy-carbon electrode and the copper center might occur via different routes.

Experimental Section

Proteins. Pseudoazurin, plantacyanin, and stellacyanin were isolated from *A. cycloclastes* IAM 1013,[10] cucumber peeling,[11] and Chinese lacquer latex[12] according to literature methods, respectively. Takano and Co., Kanazawa, Japan, supplied us with lacquer latex of the highest quality. Isolated proteins were confirmed to be pure by electrophoresis. Absorption ratios of the band at about 280 nm and the charge-transfer band (S^-(Cys) → Cu^{2+}) at about 600 nm were 1.5 for pseudoazurin and 6 for both plantacyanin and stellacyanin.

Blue copper proteins at different pH's were prepared by dialyses for 1 day against an appropriate-pH 0.1 M potassium phosphate buffer. Protein concentrations were determined on the basis of the absorbance at 280 nm. All chemicals were used without further purification.

Measurements. Cyclic voltammetry was carried out by using a Yanagimoto P-1100 voltammetric analyzer or a Bioanalytical Systems (BAS) CV-1B cyclic voltammetry unit. A glassy (vitreous) carbon disk (Tokai Carbon, GC-20) with a surface area of 0.30 cm^2, a platinum wire, and a saturated calomel electrode were used as the working, counter, and reference electrodes, respectively. Details concerning the structure of the glassy carbon were not provided. The material is, however, isotropic, as opposed to pyrolytic graphite, which is ansiotropic. Measurements were made at room temperature (ca. 25 °C) under an argon atmosphere for aqueous protein solution with 0.1 M potassium phosphate buffer as a supporting electrolyte. The scan rate was 1 mV/s. Prior to each ex-

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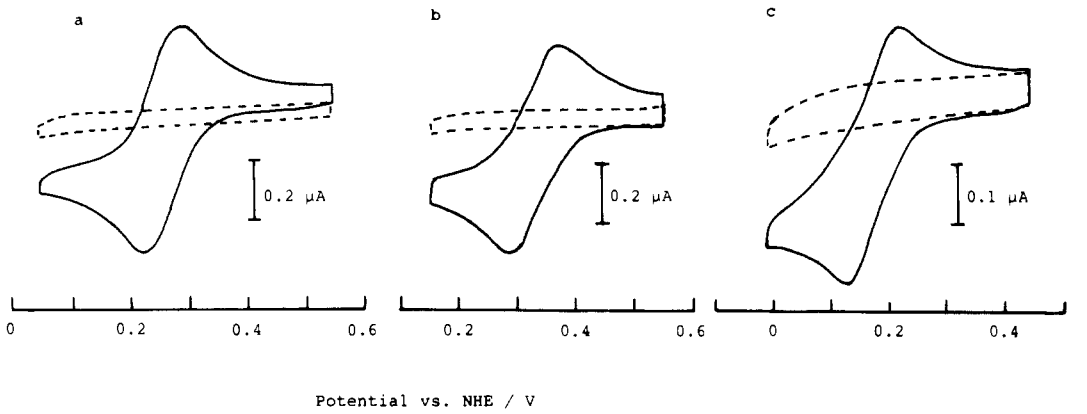

Figure 1. Cyclic voltammograms of (a) pseudoazurin (pH 8.0), (b) plantacyanin (pH 6.0), and (c) stellacyanin (pH 7.6) recorded in 0.1 M potassium phosphate buffer (sweep rate 1 mV/s, ca. 25 °C).

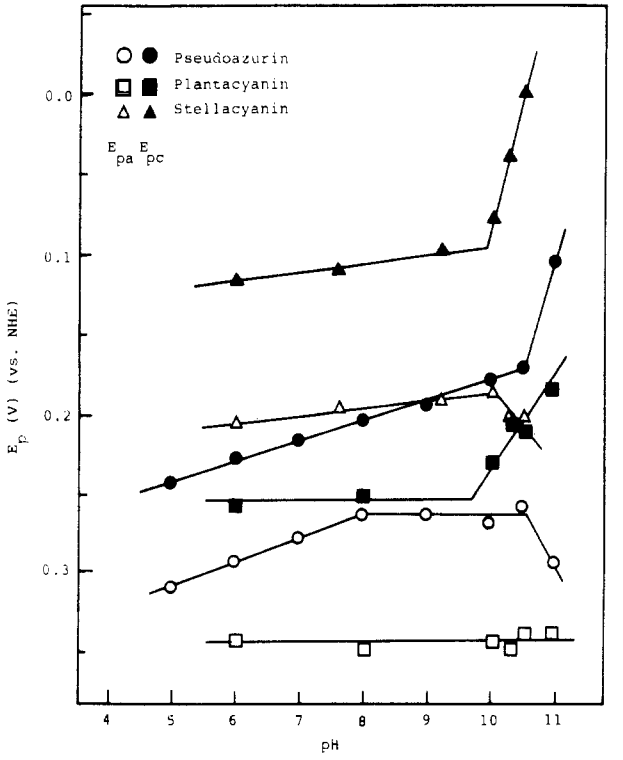

Figure 2. pH dependencies of the anodic (○,□,△) and cathodic (●,■,▲) peak potentials of pseudoazurin, plantacyanin, and stellacyanin.

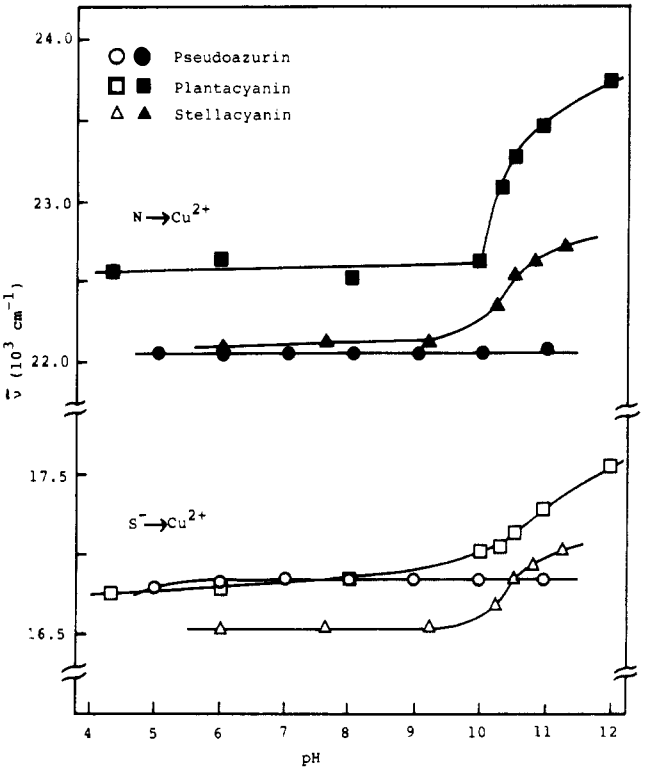

Figure 3. pH dependencies of the S^-(cys) → Cu^{2+} (○,□,△) and N(His) → Cu^{2+} (●,■,▲) charge-transfer bands of pseudoazruin, plantacyanin, and stellacyanin.

periment, the electrode was polished with alumina powder (0.3 μm) and cleaned in an ultransonic bath.

Absorption spectra were measured on a JASCO Ubest-50 spectrometer at room temperature. ESR spectra were obtained on a JEOL JES-RE1X X-band spectrometer at 77 K.

Results and Discussion

Electrochemistry of Blue Copper Proteins. Cyclic voltammograms of three blue copper proteins at a glassy-carbon electrode are illustrated in Figure 1. Well-defined cathodic and anodic waves (with peak separations (ΔE_p) of 60 mV for pseudoazurin at pH 8.0, 85 mV for plantacyanin at pH 6.0, and 86 mV for stellacyanin at pH 7.6) are generated at the bare glassy-carbon electrode. The half-wave potentials did not change on repeated runs. The scan rate dependence of the peak currents suggested that the electron transfer is dominated by diffusion of species to the electrode surface. It has been reported that plastocyanin gives well-defined waves on addition of a cation, Mg^{2+} [7a] or $[Pt(NH_3)_6]^{4+}$.[7b] Cyclic voltammetry of rusticyanin has been accomplished on a 4,4′-bipyridyl-modified carbon-paste electrode. However, direct electrochemistry of the present three basic blue copper proteins has been performed in the absence of a mediator, promoter, and modifier. In line with this, cucumber plastocyanin gave no redox wave without $MgCl_2$.

The pH dependencies of cathodic and anodic wave potentials for pseudoazurin, plantacyanin, and stellacyanin are shown in Figure 2. Cathodic and anodic peak currents for each pair of redox waves were almost the same. The formal potentials ($E^{\circ\prime}$) of pseudoazurin, plantacyanin, and stellacyanin at pH 6 were 0.28, 0.32, and 0.18 V (vs NHE), respectively. The potentiometric values of pseudoazurin, plantacyanin, and stellacyanin have been reported to be 0.245,[10a] 0.317,[9a] and 0.184 mV[9b] (vs NHE) at pH 7, respectively. Therefore, the formal potentials determined by the present direct electrochemical study appear to be extremely reliable.

Cyclic voltammetry affords not only redox potential values but also thermodynamic and kinetic information.[7b] As shown in Figure 2, the pH dependencies of the redox behaviors for three blue copper proteins are markedly diverse. In the case of pseudoazurin, the cathodic peak potential shifts linearly in the negative direction when the pH is raised to 10.5 and shifts abruptly at higher pH's. Although the anodic peak potential shifts linearly in the negative direction when the pH is raised to 8, it does not change between pH 8 and 10.5 and begins to shift in the positive direction at higher pH's. The ΔE_p value is smallest at pH 8 (60 mV), indicating that the oxidation–reduction process is almost completely reversible

and the rate of the electron transfer between pseudoazurin and the glassy-carbon electrode is extremely rapid. In the case of plantacyanin, the cathodic peak potential does not change between pH 6 and ca. 9.5 but begins to shift in the negative direction at pH >9.5. On the other hand, the anodic peak does not show pH dependence throughout the pH range of study. The cathodic peak of stellacyanin exhibits pH dependence similar to that shown by pseudoazurin. Although the anodic peak of stellacyanin shows a negative potential shift with rising pH, a positive potential shift is observed at pH >10.

The E_p values (60–90 mV) indicate that the redox process is almost reversible or quasi-reversible at pH <10. Preliminary standard heterogeneous rate constants ($k°$) for pseudoazurin, plantacyanin, and stellacyanin at pH 6.0 are 1.69×10^{-3}, 0.38×10^{-3}, and 0.33×10^{-3} cm/s, respectively. These values are comparable with those of plastocyanin at pH 8, 2×10^{-3} cm/s, and cytochrome *c* at pH 7, 6×10^{-3} cm/s).[7b] The rate ($k°$) for pseudoazurin at pH 8.0 is so rapid that it cannot be estimated by cyclic voltammetry. The present results indicate that each blue copper protein gives the smallest ΔE_p value at an intrinsic pH: pH 8.0 for pseudoazurin, pH 6.0 for plantacyanin, and pH 7.6 for stellacyanin. Nevertheless, it is not certain whether the pH value where the smallest ΔE_p value is realized is the optimal pH of the electron transfer in vivo. In addition, comparing the rates of electron transfer at a certain pH (usually pH 7.0) does not seem to be necessarily meaningful.

Absorption Spectra. The pH dependencies of the absorption spectra were investigated in order to compare with those of the electrochemical behavior. Figure 3 shows the absorption maxima of the charge-transfer bands: S^-(Cys) → Cu^{2+} at $(16.5–17.5) \times 10^3$ cm^{-1} and N(His) → Cu^{2+} at $(22 - 24) \times 10^3$ cm^{-1}. Both of the charge-transfer bands arising from plantacyanin and stellacyanin exhibit prominent blue shifts at pH >10. Such behavior is largely harmonious with the anodic and cathodic peak shifts shown in Figure 2. The pH dependencies may be associated with a change in the protein structure derived from deprotonations of the amino acid residues, Tyr (OH), Lys (NH_3^+), His (NH), and Arg (guanidinium group). Plantacyanin contains 4 Tyr, 6 Lys, 2 His (ligands on copper), and 3 Arg,[9a] and stellacyanin, 6 Tyr, 10 Lys, 4 His (2 His are ligands on copper), and 1 Arg.[13] Deprotonation from several amino acid residues, which is reflected in the charge-transfer bands of N, S^- → Cu^{2+}, is considered to be the primary factor in modifying the electrochemical behavior of the blue copper proteins. However, the absorption spectrum of pseudoazurin does not show pH dependence over the pH range 6–~10, although the protein cotains 3 Tyr, 11 Lys, 2 His (ligands on copper), and 1 Arg.[12] In the case of this protein, the reduced form might be affected by the deprotonation from several amino acid residues and the electrochemical behavior exhibits the pH dependence, especially at high alkaline pH.

ESR Spectra. In the ESR spectra, plantacyanin takes two forms: the neutral one and the high alkaline one.[11b,14] A subtle modification in the $g_\parallel$ region was noticable, as reported previously (spectra not shown). The $g_\parallel$ component of stellacyanin also began to deform with increasing pH (spectra not shown). It has been reported that at an extremely high alkaline pH Cu^{2+} is dissociated from its intrinsic ligand groups to give a biuret complex.[15] On the other hand, the ESR spectrum of pseudoazurin did not show pH dependence (spectra not shown). This fact indicates that the electronic structure of the Cu^{2+} center is not necessarily the major factor for modifying the redox behaviors.

Electron-Transfer Process. Long-range electron transfer of several blue copper proteins has been studied by the stopped-flow method,[16] the temperature jump method,[17] flash photolysis,[18] and pulse radiolysis.[19] Kinetic studies using ruthenium-derived derivatives[16g,18a] and those using substitution-inert Cr(III) as a redox couple facilitated discussion of entry and exit of an electron into and from the copper center.[20] According to those studies, two sites, a surface patch located around the ligated histidine imidazole group and a remote aromatic group from the active site (~12 Å), have been suggested for exit and/or entry of an electron. Generally, cathodic and anodic waves simply begin to separate in a similar manner with decreasing reversibility. The observed unique pH dependencies of the oxidation and reduction waves (Figure 2) may be realized, when the mechanism in which an electron reaches the central Cu^{2+} ion and the mechanism in which an electron comes out from the Cu^+ ion to the exterior of the protein are different. Although no kinetic study on plantacyanin and pseudoazurin has been done heretofore, both blue copper proteins may have two reactive sites as have been supposed for plastocyanin, azurin, and stellacyanin.[1,2d,21] In addition, the slopes of the shifts of the anodic and cathodic waves are much smaller than −59 mV/pH unit at least at pH <10. This fact strongly seems to suggest that the electron-transfer processes between the glassy-carbon electrode and the present three blue copper proteins are not accompanied by H^+ transfer. On the other hand, the H^+ release from some amino acid residues might merely complicate the situation considerably, leading to the diverse pH dependencies of E_{pc} and E_{pa} and to the slopes of $E_{1/2}$ being less than −59 mV/pH unit. Contrary to this, no conformational change was observed for the CD spectra in the UV region (data not shown). However, further study of adsorption, especially at high pH, will be required to lead to a profound understanding of the redox behavior. It has been reported that the apparent $E_{1/2}$ value of plastocyanin at decreasing low pH increases by ca. 55 mV/pH unit, with pK_a ~5.5.[22] The pK_a can be linked with dissociation of a ligating imidazole group to yield a 3-coordinated Cu(I) that is unstable for facile oxidation to 4-coordinated Cu(II).[23] On the other hand, it can be considered that protonation of the reduced form is manifested merely as an increase in the redox potential.

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In the electrochemistry of plastocyanin, $MgCl_2$ was required to realize the direct electrode reaction.[7] Consequently, the present study is the first direct electrochemistry of blue copper proteins at an untreated glassy-carbon electrode in the absence of mediator, promoter, or additive. This success may be due to the fact that the present three blue copper proteins carry a large overall positive charge (The p*I* values of pseudoazurin, plantacyanin, and stellacyanin are 8.9, 10.6, and 9.9, respectively).[9a,12,13] Very recently, Armstrong et al.[24] proposed a microscopic model for the electrochemistry of cytochrome *c*, plastocyanin, and ferredoxin at an edge- and basal-plane graphite electrode. The new model assumes that electron transfer occurs at arrays of oxygen-containing functional sites of microscopic dimensions. Oxygen-containing sites generated by surface abrasion (polishing) may facilitate the access of basic blue copper proteins, although the total charge on the glassy-carbon electrode is supposed to be positive under the present experimental conditions.[25] The combined effect of a local protein structure change by deprotonation from several amino acid residues and an electronic state change in the cupric and cuprous ions would result in the present diverse pH dependence for the redox behavior. The primary factor will differ for each protein and pH. Flash photolysis of the three blue copper proteins is under investigation.

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Iron Porphyrin Models of Peroxidase Enzymes: Catalytic Activity, Regeneration, and Oxidative Degradation of Mesoferriheme

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The reaction of the iron(III) complex of mesoporphyrin IX, mesoferriheme (mfh), with two-electron oxidants, such as sodium hypochlorite and *m*-chloroperoxobenzoic acid, produces a spectroscopically distinct oxidized heme species viewed as a functional analogue of the reaction intermediate obtained by oxidation of hemoprotein peroxidase enzymes. Unlike the peroxidase reaction product, which is the result of a 1:1 stoichiometric interaction between enzyme and oxidant, however, the oxidized mfh species displays a stoichiometric equivalence of 2 mol of heme Fe(III)/mol of two-electron oxidant. This is consistent with previously reported results on the oxidation of deuteroferriheme (dfh) and is attributed to a two-electron oxidation of monomeric heme followed by comproportionation with the free-heme Fe(III) monomer to form a dinuclear oxidation product. Second-order rate constants for the oxidation of mfh are comparable to those previously reported for dfh. Thus, although substitution of ethyl groups for hydrogen in the 2- and 4-positions of the porphyrin ring system has been shown to increase the degree of heme dimerization, it has a negligible effect on the rate of intermediate formation. Such substitution, however, does appear to increase the susceptibility of the porphyrin ring to oxidative degradation. Studies of the heme and oxidant concentration dependence of degradation suggest that porphyrin ring cleavage results from attack of the oxidant on the initially formed two-electron-oxidation product depicted as $Fe^{V}O$ and that degradation may be hindered by the scavenging action of the heme dimer on $Fe^{V}O$ with consequent comproportionation. The oxidized mfh species displays peroxidatic activity through biphasic oxidation of phenol and also undergoes biphasic in situ reduction, regenerating free mesoferriheme through processes thought to involve consecutive one-electron reductions of dinuclear analogues of enzyme compounds I and II. The formation of such dinuclear species probably relates to the tendencies of both mfh and dfh to undergo aggregation in contrast to coproferriheme, which has been reported to exist predominantly in monomeric form and to produce mononuclear oxidation products.

Introduction

Inasmuch as the catalytic activity of hemoprotein peroxidase enzymes involves redox chemistry of an iron(III) porphyrin IX or ferriheme prosthetic group,[1] a number of protein-free hemes have been studied as peroxidase models. In the absence of protein, however, ferrihemes undergo dimerization in aqueous solution[2-5] with a consequent decrease in peroxidase-like activity. The extent of dimerization is pH and concentration dependent[3] and also varies markedly with the nature of the substituents in the 2- and 4-positions of the porphyrin ring, decreasing in the series proto- > meso- > deutero- > coproferriheme[6] (figure 1). Since the reactivity of deuteroferriheme (dfh) is qualitatively similar to that of protoferriheme (pfh), and since it is readily prepared by treatment of pfh with molten resorcinol,[7] the relatively low degree of dimerization of dfh has commanded its widespread use as a peroxidase model system.[8-20]

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