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SPECTROSCOPIC AND ELECTROCHEMICAL STUDIES ON THE FOUR-α-HELICES BUNDLE CYTOCHROME *C'*

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Abstract The cyclic voltammogram of the cytochrome *c'* from *Achromobacter xylosoxidans* NCIB 11015 displayed a quasi-reversible electron-transfer process and the observed half-wave peak potential was estimated to be $E_{1/2}$ = 184 mV *vs* NHE. The redox potential of cyt *c'*(Fe^{3+} / Fe^{2+}) decreases with increasing buffer concentration, implying that the oxidized state of the protein is stabilized by counter anion shielding of the positive charges on the protein surface. The intensities of the magnetic circular dichroism (MCD) spectra of the protein at λ = 402 and 418 nm decrease with increasing ionic strength. The decreasing of the MCD intensity at high ionic strength indicates the increasing of the high-spin state species. These results suggest that the structural change of the solvent exposed charged amino acid residues linking to outside of the protein might allow the regulation of the electron-transfer reaction of cytochrome *c'*.

INTRODUCTION

Cytochromes *c'* are a class of c-type cytochrome, which have been found in several photosynthetic bacteria and denitrifying bacteria.[1] Cytochrome *c'* form denitrifying bacteria is a positively charged protein containing a heme prosthetic group covalently bound to the protein backbone through two thioether linkages as well as cytochrome *c*.[2]

Three dimensional structural studies of cytochrome *c'* show that a histidine imidazole is coordinated to iron center from the 5th position, and the 6th position is vacant.[3-7] The heme prosthetic group of cytochrome *c'* shows high-spin character like hemoglobin and myoglobin. The characteristic magnetic behavior of ferric cytochrome *c'* have been proposed the quantum mechanical admixture of two spin states, intermediate spin state (S = 3/2) and common high spin state (S = 5/2).[8-11] Spectroscopic studies of cytochrome *c'* and its derivatives have been discussed.[12-14] The EPR spectroscopic studies of cytochrome *c'* from denitrifying bacteria indicate that the ground state of the ferric protein is in an almost high spin state due to the very small contribution of intermediate spin state.[15] Kitner and Dawson have reported near-infrared (NIR) MCD spectra of ferric porphyrins. The NIR MCD spectra of Fe(OEP)X (X = ClO_4, SO_3CF_3,

SbF_6) and $[Fe(OEP)(3,5\text{-}Cl_2Py)_2]^+$, all of which contain an admixed S = 3/2, 5/2 ground state, are unique. They have reported the close similarity of NIR MCD of cytochrome *c'* spectra with those model complexes.[16] Suzuki et al. have demonstrated the MCD spectra of nitrosylhem derivatives of cytochrome *c'*, which are substantially identical with those of the pentacoordinate nitrosyl(protophorphyrin IX dimethyl esterato) iron (II) complex.[14] They also reported the spectroscopic properties of CO and CN^- cytochrome *c'* complexes. The CO affinity of cytochrome *c'* from denitrifying bacteria is less reactive than those of most photosynthetic bacteria. The formation of ferrous CN^- complex of cytochrome *c'* was identified spectroscopically.[13]

In contrast, a few direct electrochemistry of cytochrome *c'* have been reported. Erabi et al. reported the direct electrochemistry of *Rhodospirillum rubrum* cytochrome *c'*, which showed a well-defined cyclic voltammetric behavior at a 2-mercaptosuccinic acid modified gold electrode, and the observed peak-to-peak separation between reduction peak potential and oxidation peak potential was reported to be 100 mV.[17]

In the present study, we describe the spectroscopic and the electrochemical behavior of a four-α-helices bundle cytochrome *c'* form a denitrifying bacterium *Achromobacter xylosoxidans* NCIB 11015.

ELECTROCHEMISTRY OF CYTOCHROME *C*

The cyclic voltammogram of cytochrome *c'* exhibits a well-defined electrochemical response indicating a fast electron-transfer at a 6-mercaptopurine modified gold (6mp / Au) electrode, but not at a bare gold electrode. The cyclic voltammograms of cytochrome *c'* at different scan rates are illustrated in Figure 1. At a 6mp / Au electrode (pH 7.0, 10 mM Tris-HCl buffer),[18] the cyclic voltammogram of cytochrome *c'* indicated a quasi-reversible electron-transfer process and the observed half-wave peak potential was estimated to be $E_{1/2}$ = 184 mV *vs* NHE (ΔE_p = 78 mV). The cathodic peak current (i_{pc}) was found to increase linearly with the square root of the potential scan rate ($v^{1/2}$), as expected for a diffusion-controlled process.[19] The diffusion coefficient was estimated to be 8.3×10^{-7} cm^2 s^{-1} at pH 7.0 from the slope of the plot i_{pc} against $v^{1/2}$. The heterogeneous electron-transfer rate constant (k_S) of the protein at the 6mp / Au electrode was estimated to be 3.1×10^{-4} cm s^{-1} at pH 7.0 (10 mM Tris-HCl). The peak-to-peak separation for the cyclic voltammograms of cytochrome *c'* was broadened with increasing ionic strength, and indistinct voltammogram of the protein was obtained at 400 mM ionic strength.

The redox potential of cytochrome *c'* (Fe^{3+} / Fe^{2+}) decreases with increasing buffer concentration (Figure 2). It is implied that the oxidized state of the protein is

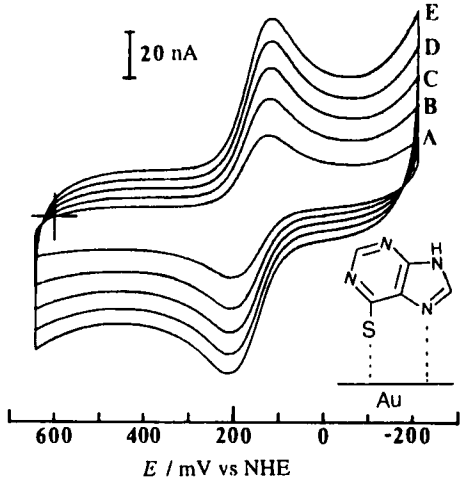

FIGURE 1 Cyclic voltammograms of cytochrome *c'* at 6mp /Au modified electrode (pH 7.0, 10 mM Tris-HCl buffer); (A) 20, (B) 40, (C) 60, (D) 80, and (E) 100 mV /s.

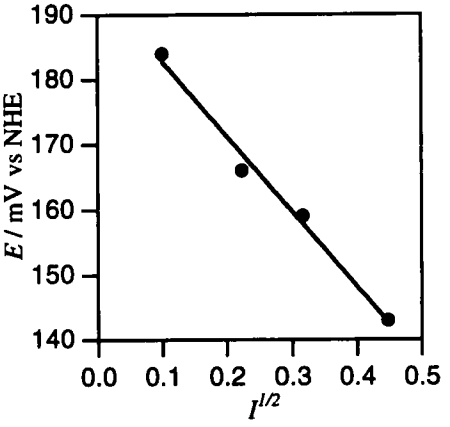

FIGURE 2 Observed redox potentials of cytochrome *c'* as a function of ionic strength.

stabilized by counter anion shielding of the positive charges on the protein surface,[20] and / or the distance between solvent exposed histidyl imidazole ligand and the iron center is changed as an ionic strength effect. Model complexes of ferric octaethylporphyrin (OEP)[21] and tetraphenylporphyrin (TPP)[22] having a weak axial ligand have been synthesized and demonstrated that indicate admixed intermediate spin behavior like cytochrome *c'*. The reduction potential for the intermediate spin state, $Fe(TPP)ClO_4$ ($E_{1/2}$ = 0.14 V *vs* SCE) is positive than that of the usual chloride high-spin derivative ($E_{1/2}$ = -0.29 V *vs* SCE). The reduction potential for the S = 3/2, 5/2 spin admixture, $Fe(TPP)SO_3CF_3$ is $E_{1/2}$ = 0.08 V *vs* SCE.[23] The redox potentials of these model complexes might indicate that the structural rearrangements of cytochrome *c'* are related to the spin state, and the shielding effect of counter anion also modulates the redox potential of the protein. Cytochrome *c'* shows a faster electron transfer process at low ionic strength and slower electron transfer at high ionic strength. In the case of electrochemical experiments of pseudoazurin, which is a copper containing electron-transfer protein, fast electron transfer of the protein is believed to be achieved by hydrogen bonding between the lone-pair electrons on a pyridine moiety of bis(4-pyridyl)disulfide modified gold electrode and positively charged lysine residues on pseudoazurin[24] and cytochrome c.[25] In the molecular structure of cytochrome *c'*, positively charged amino acid residues are located near the both solvent exposed phenylalanine residues and heme active site. The high ionic strength conditions inhibit the interaction of hydrogen bonding between the purin moiety of the 6mp / Au electrode

and cytochrome *c'*. The purine moiety of the 6mp / Au electrode might be possible to make a hydrophobic interaction with the aromatic rings of the solvent exposed phenylalanine residues and hydrogen bonding with the positively charged amino acid side chains.

ELECTRONIC ABSORPTION SPECTRA

The electronic absorption spectra of cytochrome *c'* at various ionic strength are illustrated in Figure 4. The ionic-strength dependence was investigated in two different media: one was a Tris-cacodylate buffer as the "non-binding" medium and the other medium was Tris-HCl buffer, however, no significant difference was observed between Tris-cacodylate and Tris-HCl buffer. The molar extinction coefficients at the Soret band of the protein decrease with decreasing ionic strength, and the shoulder band at λ = 380 nm appeared clearly at low ionic strength. The Soret band at λ = 403 nm (10 mM Tris-Cacodylate) slightly shifted to shorter wavelength, λ = 400 nm (400 mM Tris-Cacodylate). The Soret bands for Fe(TPP)Cl (pure high-spin state complex), $Fe(TPP)SO_3CF_3$, and $Fe(TPP)ClO_4$ are located at 402, 408, and 415 nm, respectively.[23] Consequently, the electronic absorption spectra of cytochrome *c'* at low ionic strength suggest much intermediate spin state contribution.

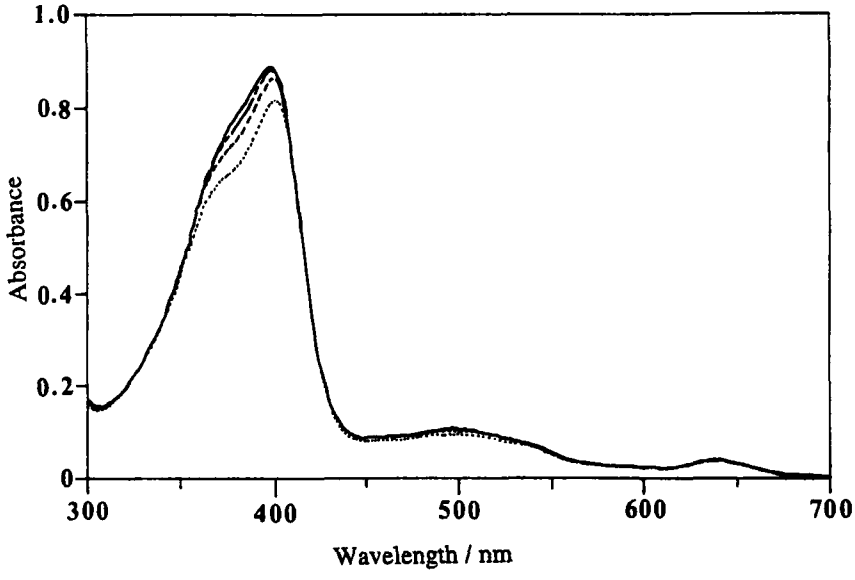

FIGURE 3 Electronic absorption spectra of cytochrome *c'* (11 μM) at pH 7.0 and at 400 mM (solid), 100 mM (broken), 50 mM (dashed), and 10 mM (dotted) Tris-HCl buffer concentration.

MAGNETIC CIRCULAR DICHROISM SPECTRA

A number of magnetic circular dichroism (MCD) spectroscopic studies of heme iron systems have demonstrated the utility of the technique as a probe of spin state.[26] High-spin ligand adducts of ferric myoglobin display much weaker Soret MCD signals than that of low-spin state. The MCD signals in this region are dominated by *C* terms which are sensitive to both spin state and temperature. The plots of MCD intensity against ionic strength are represented in Figure 5. The intensity of the MCD spectra of the protein at λ = 402 and 418 nm decreases with increasing ionic strength. The increasing of the MCD intensity of the Soret band at high ionic strength indicates the increasing of the high-spin state species.[16] Therefore, the intermediate spin state of cytochrome *c'* increases at low ionic strength.

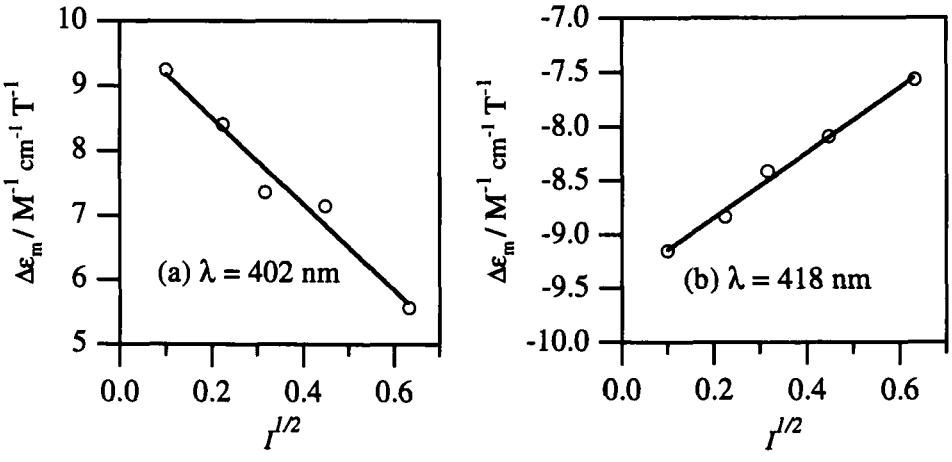

FIGURE 4 Plots of the MCD intensities against ionic strength at two different MCD absorption bands, (a) 402 nm and (b) 418 nm.

CONCLUSION

Cytochrome *c'* indicates ionic-strength dependent structure rearrangements. The high spin state of the protein decreases and the intermediate spin state increases at low ionic-strength. The variation of MCD and electronic absorption spectra at different ionic strength may be interpreted that the structural change occurs around heme active site involving solvent exposed phenylalanine aromatic rings and positively charged amino acid residues. The direct electrochemistry at the 6mp / Au electrode demonstrates the possibility of interaction modes, which are hydrophobic aromatic-aromatic interactions

and hydrogen bonding of cytochrome *c'* with the electrode surface. These results suggest that the structural change of the solvent exposed moiety linking to outside of the protein might allow the regulation of the electron-transfer reaction of cytochrome *c'*.

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