Electrochemistry of Horse Heart Cytochrome *c*

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Abstract: The electrochemical behavior of horse heart ferricytochrome *c* at a gold electrode in the presence of 4,4′-bipyridyl is described. DC and AC cyclic voltammograms are obtained due to the quasi-reversible electrode reaction of cytochrome *c*, which has $E_{1/2}{}^{r} = +0.255$ V vs. NHE. Combined coulometric and spectrophotometric studies confirm that the one-electron reduction is due to the cytochrome. 1,2-Bis(4-pyridyl)ethylene is also effective in promoting electron transfer. The results are consistent with adsorption of the heterocycle to form a conducting layer on the gold surface. Variation of the AC cyclic voltammetry with pH indicates the existence of an electroactive and an electroinactive form of cytochrome *c* at alkaline pH while at acidic pH protonation of 4,4′-bipyridyl appears to affect the observed electrochemistry.

Cytochrome *c* is a heme protein, widely distributed in living organisms, whose function is electron transfer in the mitochondrial respiratory chain. The mechanism of reduction and oxidation of the protein heme iron is therefore of great interest. However, application of conventional electrochemical techniques, voltammetry and coulometry, to the study of its redox behavior has enjoyed only limited success. For example, the direct electrochemical reduction of horse heart ferricytochrome *c* at the mercury electrode was shown[1-3] to take place irreversibly in the electrochemical sense. Similarly at the platinum electrode the rate of reduction of cytochrome *c* was found[4] to be very slow at potentials more positive than −1.0 V with respect to the saturated calomel electrode (SCE), the reduction giving rise to reduced cytochrome *c*, inactivated with respect to reaction with cytochrome a_3. We showed[5] cytochrome *c* to be electroinactive at a gold electrode at potentials close to its standard electrode potential, E^0. Thus electrochemical studies of cytochrome *c*, and metalloproteins in general, have had to rely on the use of indirect methods. This typically involves the use of added mediators[6,7] to effect electron transfer between the electrode and the protein. While giving useful thermodynamic information, such methods provide little kinetic data. Kuwana has recently shown[8] that cytochrome *c* exhibits reversible electron transfer characteristics at a tin-doped indium oxide electrode. As we recently reported,[5] cyclic voltammograms of cytochrome *c* are obtained at a gold electrode in the presence of 4,4-bipyridyl, corresponding to a quasi-reversible one-electron process. From this we determined a value for the half-wave potential, $E_{1/2}{}^{r}$, of +0.25 V with respect to the normal hydrogen electrode (NHE). This is in excellent agreement with previously determined[6,7] values of E^0 for cytochrome *c*. It appears[2,3,9] that the nature of the electrode/solution interface is a critical factor in the electron-transfer reactions of metalloproteins, and that a suitable interface can be obtained by using semiconductor[8] rather than metal electrodes, or by modification of the gold electrode surface in the presence of 4,4′-bipyridyl. 4,4′-Bipyridyl, a molecule well-known[10-12] for its ability to promote electron transfer in polynuclear transition metal complexes, may act by formation of an adsorbed conducting monolayer at the electrode surface, modifying the electrode surface in such a way that rapid electron transfer to cytochrome *c* can take place. We describe here further experiments on the electrochemistry of cytochrome *c* in the presence of 4,4′-bipyridyl and other nitrogen heterocycles.

Experimental Section

Electrochemical Measurements. Dc cyclic voltammograms were obtained using a PAR 173/179 potentiostat with a dc ramp generator built in this laboratory and were recorded on a Bryans *XY* recorder 26000 A3. Ac fundamental and second harmonic voltammograms were obtained using the above equipment in conjunction with a sine wave voltage generator, also built in this laboratory, and an Ortec Brookdeal lock-in amplifier 9501. The cyclic voltammetry cell was of all-glass construction, approximately 1 mL in volume, incorporating a conventional three-electrode system. The working electrode consisted of a gold disk, 2 mm in diameter, mounted in the end of a glass rod and sealed in with epoxy resin. Before each experiment it was polished using fine alumina suspension and then washed with distilled water. The secondary electrode was a platinum gauze and the reference electrode was the saturated calomel electrode. The reference electrode was connected to the cell via a luggin capillary tip and to the potentiostat via a PAR Model 178 electrometer probe. The controlled potential reduction was carried out using a PAR 173/179 potentiostat in an all-glass cell, 10 mL volume, with a conventional three-electrode system. The working electrode consisted of a cylinder of gold-plated platinum gauze supplied by Johnson Matthey Ltd., Reading, England, which was cleaned with hot nitric acid prior to the electrolysis. The secondary electrode was a platinum gauze contained in a Corning Vycor test tube, occupying a position at the center of the cylindrical working electrode. As with the voltammetry calomel was used as a reference. Stirring of the solution was achieved using a magnetic stirrer in the bottom of the cell. A Cary Model 17 spectrophotometer was used for all optical measurements.

Materials. Horse heart ferricytochrome *c* type VI was obtained from the Sigma Chemical Co. It was further purified to remove small amounts of polymeric and deamidated forms by ion-exchange chromatography using diethylaminoethylcellulose resin (DE 32, Whatman Biochemicals Ltd., England) as described[13] by Brautigan et al. 4,4′-Bipyridyl, 2,2′-bipyridyl, and pyridine were obtained from BDH and 1,2-bis(4-pyridyl)ethylene, 1,2-bis(4-pyridyl)ethane, pyrazine, and 4-phenylpyridine were obtained from the Aldrich Chemical Co. All other chemicals used were Analar grade, and all solutions were made up with doubly distilled water. All solutions were degassed with O_2-free argon before use.

Assay Procedure. Ascorbate–*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine oxidase activities with various cytochrome *c* concentrations were measured as described[13] using Keilin–Hartree particles (KHP) depleted of cytochrome *c* prepared[14] by the method of King as modified[15] by Ferguson-Miller, with the following alterations. After mixing and washing, the sand grinding step was replaced by homogenization in a Waring-type blender at high speed for 5 min using 500 mL of 20 mM potassium phosphate (pH 7.8) per 250 g of minced, washed muscle. Method 3 of King[14] was used to sediment the KHP which were resuspended in phosphate–borate (pH 7.8) to 34 mg protein/mL. Part of the preparation stored in closed vials at 4 °C was used in these assays. KHP suspension (6 μL) used in each assay gave a final protein concentration of 0.12 mg/mL. Stock solution of cytochrome *c* was 1.013 mM in assay buffer.

Results and Discussion

Cyclic Voltammetry of Cytochrome *c*. The dc cyclic voltammogram of a solution of cytochrome *c* at pH 7 at the gold electrode in the potential range of +0.20 to −0.20 V vs. SCE in the absence of 4,4′-bipyridyl is indistinguishable from normal background, showing the absence of any electrode process under these conditions. In the presence of 4,4′-bipyridyl, dc cyclic voltammograms are obtained (Figure 1) which are due

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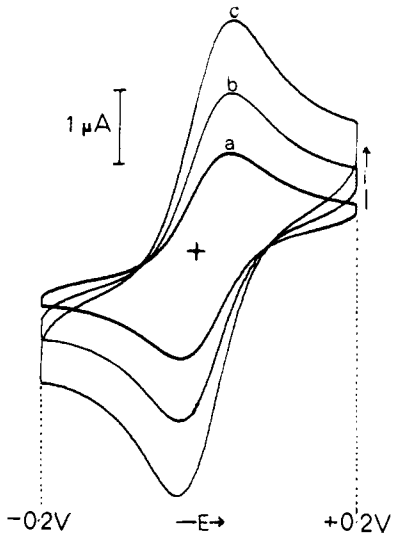

Figure 1. Dc cyclic voltammetry of horse heart ferricytochrome *c* (5 mg mL^{-1}) in $NaClO_4$ (0.1 M), phosphate buffer (0.02 M) at pH 7 in the presence of 4,4′-bipyridyl (10^{-2} M) in the potential range +0.20 to −0.20 V vs. SCE, sweep rate (a) 20 mV s^{-1}, (b) 50 mV s^{-1}, (c) 100 mV s^{-1}.

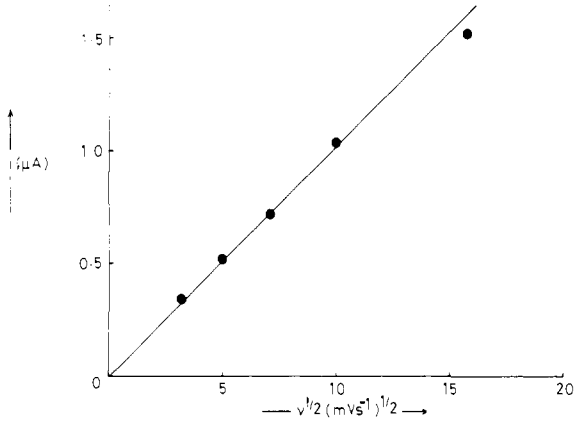

Figure 2. Plot of i_p vs. $\nu^{1/2}$ for dc cyclic voltammetry.

to a reversible electrode process. Current peaks due to the faradic process are centered around 0.0 V vs. SCE. These are attributable to the reduction and subsequent reoxidation of cytochrome *c*, the reduction of 4,4′-bipyridyl occurring at much lower potentials, about −0.9 V vs. SCE. The separation of forward (reduction) and reverse (reoxidation) peaks, ΔE_p, was found to be essentially constant at 60 mV using sweep rates of between 10 and 500 mV s^{-1}. This is consistent with the theoretical value[16] for a fully reversible, diffusion-controlled, one-electron process of 57 mV at 298 K. The midpoint between the forward and reverse peak potentials, which for a reversible process occurs[16] at the half-wave potential, $E_{1/2}{}^r$, is found at 0.01 V vs. SCE (0.255 V vs. NHE). The peak current, i_p, was found to increase linearly with the square root of the scan rate, $\nu^{1/2}$ (Figure 2), as expected[16] for a diffusion-controlled process. From the slope of i_p vs. $\nu^{1/2}$ a value for the diffusion coefficient of 9.4×10^{-7} cm^2 s^{-1} was calculated in good agreement with values (11×10^{-7} cm^2 s^{-1}) determined[17,18] by other methods. Thus the dc cyclic voltammetry shows that cytochrome *c* takes part in a rapid one-electron reaction at the 4,4′-bipyridyl modified gold electrode.

Ac fundamental harmonic cyclic voltammetry of cytochrome *c* (Figure 3) yields overlapping peaks as expected[19] for an electrode process which is sufficiently rapid to achieve Nernstian behavior in the dc sense. At low frequencies of potential modulation the summit potential, E_s, of the ac voltammogram coincides with the $E_{1/2}{}^r$ determined from the dc

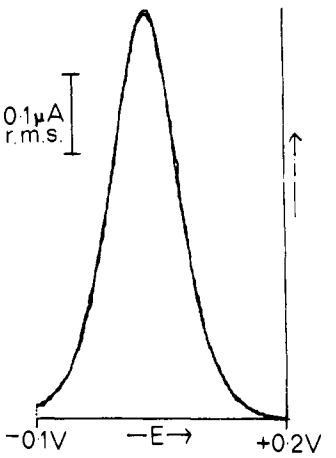

Figure 3. Ac fundamental harmonic voltammetry of horse heart ferricytochrome *c* (5 mg mL^{-1}) in $NaClO_4$ (0.1 M), phosphate buffer (0.02 M) at pH 7 in the presence of 4,4′-bipyridyl (10^{-2} M) in the potential range +0.20 to −0.10 V vs. SCE, applied potential modulation frequency 14 Hz, amplitude 8 mV.

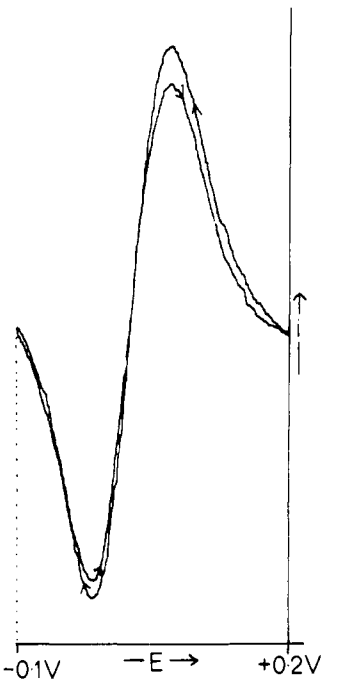

Figure 4. Ac second harmonic voltammetry of horse heart ferricytochrome *c* (5 mg mL^{-1}) in $NaClO_4$ (0.1 M), phosphate buffer (0.02 M) at pH 7, in the presence of 4,4′-bipyridyl (10^{-2} M) in the potential range of +0.20 to −0.10 V vs. SCE, applied potential modulation frequency 1040 Hz.

voltammetry and the peak has a width at half height of 90 mV as expected for the reversible one-electron electrode process.[20] At higher modulation frequencies the peak broadens, demonstrating the quasi-reversible nature[20] of the electrode process in the ac sense. Furthermore the summit potential is shifted to more negative potentials as the modulation frequency is increased, indicative of a quasi-reversible process in which the charge transfer coefficient, α, is greater than 0.5. Ac second harmonic cyclic voltammograms of cytochrome *c* in the presence of 4,4′-bipyridyl (Figure 4) are also shown.

Visible Spectroscopy of Cytochrome *c* in the Presence of 4,4′-Bipyridyl. The absorbance of cytochrome *c* in the wavelength region 500–750 nm showed no change upon the addition of 4,4′-bipyridyl to the solution. In particular the band at 695 nm, which is known[21] to disappear on complex formation with extrinsic ligands, is unaffected. From this we conclude that 4,4′-bipyridyl does not bind directly to the heme iron. Thus it

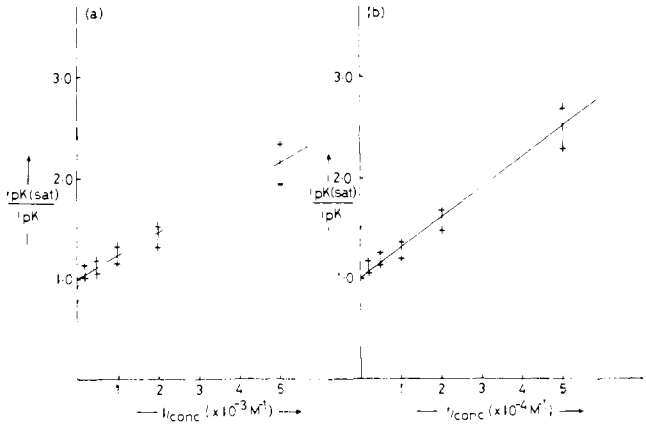

Figure 5. (a) Plot of $1/i_{pk}$ against $1/C$ for 4,4′-bipyridyl for a solution of horse heart cytochrome *c* (5 mg mL^{-1}), $NaClO_4$ (0.1 M) phosphate buffer (0.02 M), pH 7. (b) Plot of $1/i_{pk}$ against $1/C$ for 1,2-bis(4-pyridyl)ethylene for a solution of horse heart cytochrome *c* (5 mg mL^{-1}), $NaClO_4$ (0.1 M), phosphate buffer (0.02 M) at pH 7.

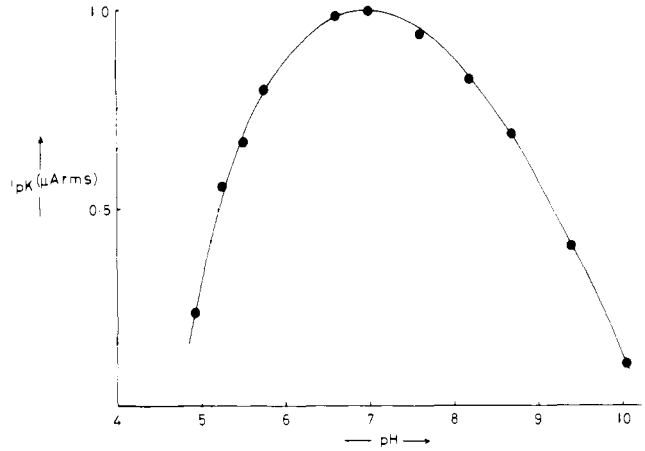

Figure 6. Plot of i_{pk} against pH for a solution of horse heart cytochrome *c* (5 mg mL^{-1}), $NaClO_4$ (0.1 M), phosphate buffer (0.02 M), and 4,4′-bipyridyl (10^{-2} M).

appears that, rather than providing a direct electron pathway to the iron, 4,4′-bipyridyl provides an electron pathway from the electrode to the surface of the cytochrome *c*.

Controlled-Potential Reduction. The controlled-potential reduction of a solution of cytochrome *c* at pH 7 was carried out using a gold-plated platinum gauze electrode in the presence of 4,4′-bipyridyl, at a potential of −0.15 V vs. SCE. The charge passed, Q, was followed throughout the reduction and the current was found to decay exponentially with time as expected. Samples were removed at various time intervals for optical absorption measurements and peaks due to ferrocytochrome *c* were observed, demonstrating the reduction of cytochrome *c*. The ratio of reduced to oxidized form was calculated from the absorbance measured at 520 and 550 nm[22] and was used to calculate a value for the number of electrons transferred per molecule of protein reduced, $n = 1 \pm 0.04$. Controlled-potential reoxidation of the electrochemically reduced cytochrome was performed at a potential of +0.15 V vs. SCE, regenerating the oxidized form which was identified by its visible absorption spectrum.

Samples were assayed after electrolysis to determine the effect on the biological activity of the cytochrome *c*. No loss of activity occurred after either complete electrochemical reduction followed by reoxidation with potassium ferricyanide or complete electrochemical reduction and electrochemical reoxidation. Therefore the electrochemical processes have no effect on the structure or chemical composition of cytochrome *c* as expected from the reversibility exhibited in the cyclic voltammetry experiments.

Assays were also carried out in the presence of 4,4′-bipyridyl and 1,2-bis(4-pyridyl)ethylene, which were found to have no significant effect on the activity of cytochrome *c*.

The reduction, carried out at a potential of −0.16 V with respect to E^0, of cytochrome *c* is rapid, giving about 50% reduction in 5 min. This compares most favorably with the slower rate observed[2] at the mercury pool electrode, 30% reduction in 2 h, where a considerably larger overpotential of about 0.3 V was used. Thus the controlled-potential reduction verifies that cytochrome *c* is reducible at the 4,4′-bipyridyl modified electrode, by a one-electron process. The greater efficiency of the reversible reduction at the modified gold electrode compared with the electrochemically irreversible reduction at the mercury electrode is shown by a faster rate of reduction at significantly lower overpotentials.

Effect of Other Nitrogen Heterocycles on Cytochrome *c* Electrochemistry. Pyridine, pyrazine, 4-phenylpyridine, 2,2′-bipyridyl, 1,2-bis(4-pyridyl)ethylene, and 1,2-bis(4-pyridyl)-ethane were tested as possible promoters of cytochrome *c* electroactivity. Of these only 1,2-bis(4-pyridyl)ethylene was found to be effective. It, like 4,4′-bipyridyl, is known[12] to promote electron transfer between transition-metal ions, presumably via the conjugated π-electron system. On the other hand, 1,2-bis(4-pyridyl)ethane, with a saturated hydrocarbon chain separating the two heterocyclic rings, which exhibits[10,12] much less effective electron transfer properties than 4,4′-bipyridyl in polynuclear transition metal complexes, did not effect cytochrome *c* electroactivity. These results support the postulate of electrode modification by formation of a conducting monolayer. The failure of pyrazine to effect cytochrome *c* electroactivity is not readily explained using this model, though the structure of the conducting monolayer may be important.

Variation of Current with Concentration of 4,4′-Bipyridyl and 1,2-Bis(4-pyridyl)ethylene. The ac cyclic voltammetry of cytochrome *c* was investigated using 4,4′-bipyridyl at concentrations between 0 and 5×10^{-3} M and 1,2-bis(4-pyridyl)ethylene at concentrations between 0 and 5×10^{-4} M. The peak current, i_{pk}, of the ac voltammetry increases sharply with increasing heterocycle concentration initially, and then tends to a limiting value at around 5×10^{-3} and 5×10^{-4} M for 4,4′-bipyridyl and 1,2-bis(4-pyridyl)ethylene, respectively. Plots of $1/i_{pk}$ against $1/C$ are linear, as shown in Figure 5. Such behavior is consistent with monolayer formation on the electrode surface, the current being proportional to the fraction of the surface covered by the heterocycle, the surface coverage being governed by the Langmuir adsorption isotherm, where the fraction covered, θ, is related to the concentration of the adsorbate, C, by $\theta^{-1} = 1 + kC^{-1}$.

Dependence of Cytochrome *c* Ac Cyclic Voltammetry on pH. The ac cyclic voltammetry of horse heart cytochrome *c* was investigated over the pH range 4.5–10.5. The peak potential remained essentially constant over this pH range while the peak current i_{pk} varied with pH as shown in Figure 6, the maximum current being observed around pH 7. Thus the ac voltammetry waves changed in magnitude though not in potential with pH nor did the kinetic characteristics change in pH.

These observations are consistent with the existence of a single electroactive form of cytochrome *c*, in a pH-dependent equilibrium with one or more electroinactive forms. Provided that the rate of interconversion between electroactive and electroinactive forms is slow with respect to the modulation frequency employed, the magnitude of the observed ac current at a given potential will reflect the concentration of the electroactive form only, while the potential and kinetic charac-

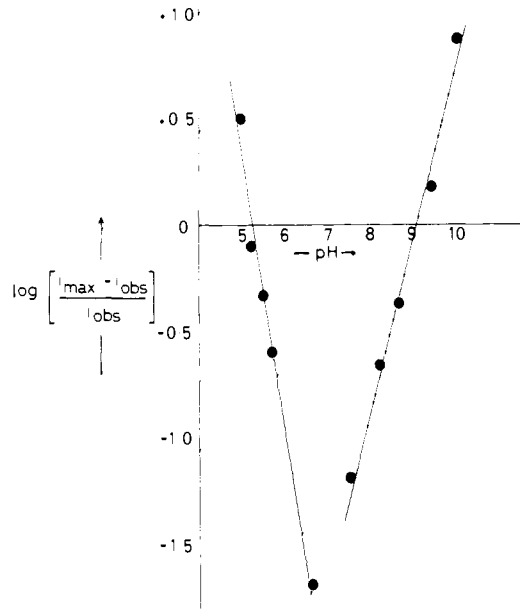

Figure 7. Plot of log $[(i_{max} - i_{obsd})/i_{obsd}]$ against pH, where i_{max} = peak ac current at neutral pH and i_{obsd} = peak ac current at the given pH. Conditions as in Figure 6.

teristics of the charge-transfer process will be those associated with the electroactive form only. Assuming the observed current to be proportional to the concentration of the electroactive form using a Henderson–Hasselbach plot, Figure 7, two pK values, 9.1 ± 0.04 and 5.25 ± 0.04, are derived. The former correlates well with a known[23] reaction of cytochrome c which has a pK value of 9.3. This reaction is associated with loss of the 695-nm absorbance band of cytochrome c and is believed to be due to replacement of the methionine residue coordinated to iron by a nitrogeneous ligand. In addition the slope of the Henderson–Hasselbach plot (Figure 7) is 0.87, consistent with the involvement of one proton in the reaction. Analysis of the results derived[24] from spectroscopic investigation leads to the same conclusion. Studies of the ascorbate reduction of cytochrome c indicate[24] the existence of an electroactive and an electroinactive form of cytochrome c at alkaline pH, again believed to be associated with the loss of 695-nm absorbance.

No known reaction of cytochrome c has a pK of 5.25. We suggest that the observed decrease in current is associated with protonation of the absorbed 4,4′-bipyridyl which has a pK_{a2} = 4.9. Protonation of the 4,4′-bipyridyl may alter its interaction with the gold surface and/or the cytochrome and experiments are in progress which may elucidate the detailed mechanism of electron transfer.

Conclusions

The results of the experiments presented here suggest that 4,4′-bipyridyl and 1,2-bis(4-pyridyl)ethylene form an adsorbed conducting monolayer on the surface of a gold electrode, enabling direct electron transfer to take place between the electrode and cytochrome c. The electrode reaction is rapid, corresponding to a quasi-reversible one-electron process as demonstrated by the dc, ac fundamental, and ac second harmonic cyclic voltammetries. A value for the reversible half-wave potential, $E_{1/2}{}^{r}$ = +0.255 V vs. NHE, is obtained from the dc and ac fundamental harmonic voltammetry. Combined coulometric and spectrophotometric studies confirm that the reduction process is the one-electron reduction of ferricytochrome c. The controlled-potential reduction occurs at a significantly faster rate and at much lower overpotentials than that previously observed at the mercury electrode, causing no loss of biological activity, and the reduced cytochrome c can be reoxidized electrochemically. The kinetics of the electrode process are found to be identical using either 4,4′-bipyridyl or 1,2-bis(4-pyridyl)ethylene, showing that the electrode process is controlled by electron transfer between the modified electrode surface and the redox center, and not by electron transfer through the heterocycle monolayer. The postulate of conducting monolayer formation is supported by the dependence of current on concentration of heterocycle where the variation in current conforms with monolayer formation in accordance with the Langmuir adsorption isotherm. We suggest that 4,4′-bipyridyl may act by preferentially adsorbing onto the electrode surface preventing adsorption of the protein and thus eliminating the problems previously encountered.[2,3]

The observed change of current with pH indicates the existence of electroactive and electroinactive forms of cytochrome c at high pH in agreement with studies of the ascorbate reduction of cytochrome c. At lower pH the current is diminished, possibly due to protonation of the 4,4′-bipyridyl.

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References and Notes

(1) L. Griggio and S. Pinamonti, *Atti Ist. Veneto Sci. Lett. Arti., Cl. Sci. Mat. Nat.*, **124**, 15 (1965–1966).
(2) S. R. Betso, M. H. Klapper, and L. B. Anderson, *J. Am. Chem. Soc.*, **94**, 8197 (1972).
(3) F. Scheller, M. Janchen, J. Lampe, H.-J. Prümke, J. Blanck, and E. Palecek, *Biochim. Biophys. Acta*, **412**, 157 (1975).
(4) T. Kono and S. Nakamura, *Bull. Agric. Chem. Soc., Jpn.*, **22**, 399 (1958).
(5) M. J. Eddowes and H. A. O. Hill, *J. Chem. Soc., Chem. Commun.*, 722 (1977).
(6) R. W. Henderson and W. A. Rawlinson, *Biochem. J.*, **62**, 21 (1956).
(7) F. M. Hawkridge and T. Kuwana, *Anal. Chem.*, **45**, 1021 (1973).
(8) P. Yeh and T. Kuwana, *Chem. Lett.*, 1145 (1977).
(9) F. Scheller, M. Janchen, and H.-J. Prümke, *Biopolymers*, **14**, 1553 (1975).
(10) J. H. Wang, *Acc. Chem. Res.*, **3**, 90 (1970).
(11) D. Gaswick and A. Haim, *J. Am. Chem. Soc.*, **98**, 5512 (1976).
(12) H. Fischer, G. M. Tom, and H. Taube, *J. Am. Chem. Soc.*, **98**, 5512 (1976).
(13) D. L. Brautigan, S. Ferguson-Miller, and E. Margoliash, *Methods Enzymol.*, **53**, 128 (1978).
(14) T. E. King, *Methods Enzymol.*, **10**, 202 (1967).
(15) S. Ferguson-Miller, D. L. Brautigan, and E. Margoliash, *J. Biol. Chem.*, **251**, 1104 (1976).
(16) R. S. Nicholson and I. Shain, *Anal. Chem.*, **36**, 706 (1964).
(17) H. Theorell, *Biochem. Z.*, **285**, 207 (1936).
(18) A. Ehrenberg, *Acta Chem. Scand.*, **11**, 126 (1957).
(19) A. M. Bond, R. J. O'Halloran, I. Ruzio, and D. E. Smith, *Anal. Chem.*, **48**, 872 (1976).
(20) D. E. Smith in "Electroanalytical Chemistry", Vol. 1, A. J. Bard, Ed., Marcel Dekker, New York, 1966, pp 1–155.
(21) A. Schejter and I. Aviram, *Biochemistry*, **8**, 149 (1969).
(22) E. Margoliash and N. Frowit, *Biochem. J.*, **71**, 570 (1959).
(23) H. A. Harbury and R. H. L. Marks in "Inorganic Biochemistry", Vol. 2, G. L. Eichorn, Ed., Elsevier, New York, 1973, p 918.
(24) C. Greenwood and G. Palmer, *J. Biol. Chem.*, **240**, 3660 (1965).
(25) T. R. Musgrave and C. E. Mattson, *Inorg. Chem.*, **7**, 1433 (1968).