1996

Electrochemical, resonance Raman, and surface enhanced Raman spectroscopic study of biomolecules: cytochrome c and its mutants

Chengli Zhou
Iowa State University

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Electrochemical, resonance Raman, and surface enhanced Raman spectroscopic study of biomolecules: Cytochrome c and its mutants

by

Chengli Zhou

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Chemistry
Major: Analytical Chemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1996

To Jianping

TABLE OF CONTENTS

GENERAL INTRODUCTION

Over the years there has been great interest in studying the electrochemical reactions of redox proteins at various electrode surfaces. Interest in this area is due to the possibility of gaining better understanding about the interactive behavior of such proteins at biological interfaces. Such studies can not only yield important information about the thermodynamics, kinetics and mechanisms of heterogeneous electron transfer reactions of the proteins at electrode surfaces, but also will provide novel insights into the electron transfer mechanisms of the proteins *in vivo*. Cytochrome c, an electron carrier between the cytochrome c reductase and the cytochrome c oxidase, has been the most widely studied of all the metalloproteins. The electrochemical reactivity of cytochrome c at bare metal electrodes such as platinum, gold, silver and mercury is often highly irreversible and in some cases undetectable, but a quasi-reversible reaction of cytochrome c can be observed at so called "promoter-modified metal electrodes" such as 4,4'-bipyridine-, bis(4-pyridyl) disulfide-, and 4-mercaptopyridine-modified gold electrodes. However, the role played by the promoters and the mechanisms of the heterogeneous electron transfer reaction of cytochrome c at electrode surfaces are not yet fully understood. In this work, conventional electrochemical methods were coupled with structure sensitive techniques, resonance Raman and surface-enhanced resonance Raman spectroscopies to study the electrochemical behavior, electron transfer mechanisms and configurations of cytochrome c at modified metal electrode surfaces. This combination can provide structural as well as thermodynamic and kinetic information about the reaction of cytochrome c at an electrode/solution interface. Four studies were undertaken. The first one reevaluated two

promoters which were studied by other research groups and a different electrode modification procedure was used. A new adsorption effect on the promoter performance was studied. Implications regarding the structural requirement for the promoter were pointed out. In the second study several organic promoters containing only one functional group were found effective in support of the ideas proposed in the first study. The third study involved investigation of the electrostatic interaction between cytochrome c and an iodide-modified electrode surface and its role in the electron transfer reaction of cytochrome c. A possible mechanism of iodide promoter effects on the electrochemical reaction of cytochrome c was suggested. In the fourth study, several cytochrome c mutants were characterized by using cyclic voltammetry, resonance Raman and surface-enhanced resonance Raman spectroscopies to investigate how the mutations affect the redox potential, electron transfer kinetics and the stability of cytochrome c, in an effort to understand more about the structure/function relationships in the redox proteins.

Dissertation Organization

This dissertation consists of a general introduction and four chapters. Immediately following the general introduction and dissertation organization are short summaries of the electrochemical behavior of cytochrome c at electrode surfaces and Raman spectroscopy of cytochrome c. References cited in the general introduction section then follow. The first chapter is a paper published in *Journal of Electroanalytical Chemistry*, Volume 319, page 71-83. The work of Chapter 2 and Chapter 3 was carried out by the doctoral candidate, Chengli Zhou, in collaboration with Professor Tianhong Lu at Changchun Institute of Applied Chemistry, Chinese Academy of Sciences. Parts of the results in

chapter 2 and 3 were published in *Chinese Chemical Letters*, Volume 3(2), page 133-134; *Bioelectrochemistry and Bioenergetics*, Volume 34, page 153-156; *Journal of Electroanalytical Chemistry*, Volume 369, page 79-86; and *Redox mechanisms and interfacial properties of molecules of biological importance*, page 56-74, Electrochemical Society, Pennington, NJ, 1993. Other authors listed on the above papers are T. M. Cotton, X. Qu, T. Lu, X. Yu and S. Dong. Chapter 4 is to be submitted to *Biospectroscopy*. A general summary concludes the dissertation.

Electrochemical Reactions of Cytochrome c at Electrode Surfaces

Cytochrome c, an electron carrier between the cytochrome c reductase and the cytochrome c oxidase, plays an important role in energy transduction in the mitochondrial respiratory chain. The primary structure of horse heart cytochrome c consists of a single polypeptide chain of 104 amino acids attached to a single iron heme unit [1]. The molecular weight of the protein is ~12400 Daltons [2]. The atom labeling scheme of the heme moiety in cytochrome c, together with a structural diagram showing the saddling distortion in the native protein is illustrated in Figure 1 [3]. The two vinyl groups of the heme become saturated by the formation of two thioether linkages with two cysteine residues 14 and 17. The iron atom is bonded to four pyrrole nitrogens in the porphyrin plane. There are two other points of attachment of the heme to the polypeptide (as shown in Figure 1) which occur at the fifth and sixth coordinate positions at neutral pH. One of these axial ligand bonds is formed with an imidazole nitrogen 18, and the other coordination site is furnished by a sulfur atom of the methionine 80.

The X-ray crystal structure of the oxidized horse heart cytochrome c has been

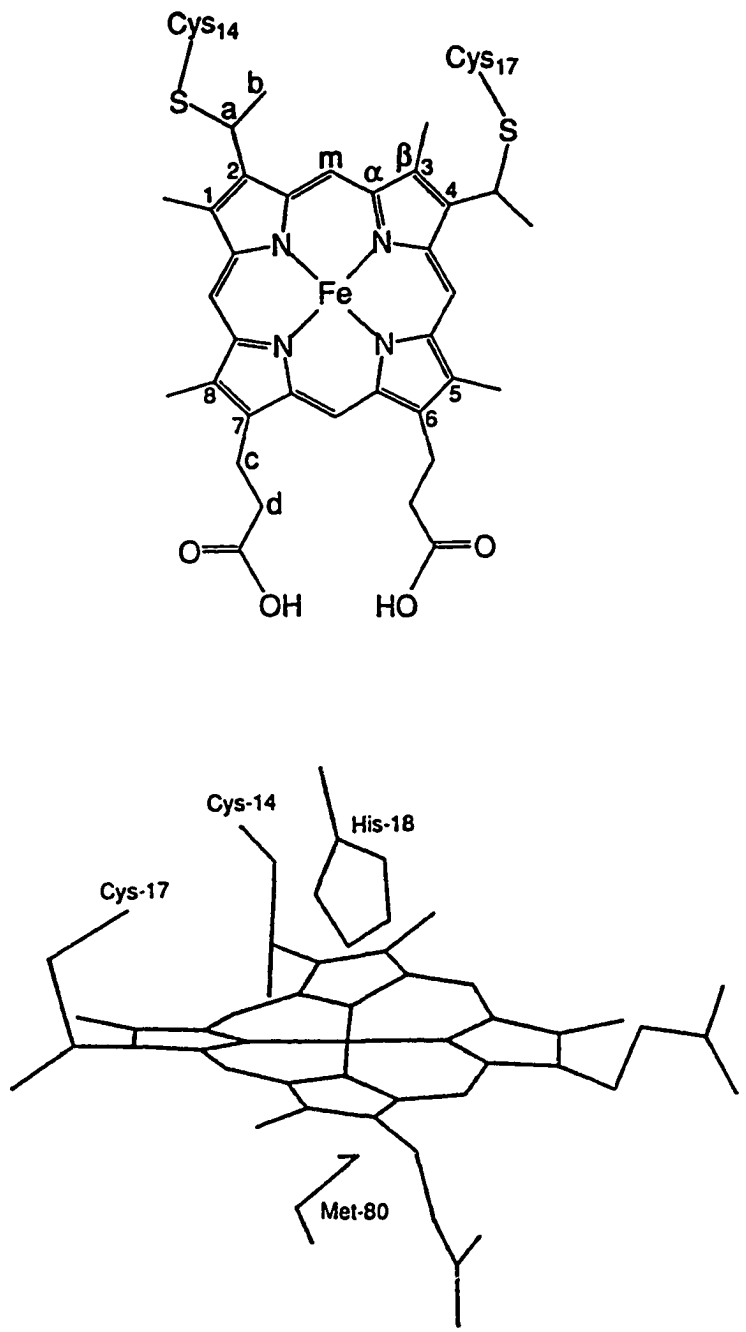

Figure 1. Atom labeling scheme of the heme moiety in cytochrome c, together with a structural diagram showing the saddling distortion in the native protein (from Ref. 3).

determined, and its α-carbon map is illustrated in Figure 2 [4]. Only those amino acids attached directly to the heme are shown in Figure 2. The other amino acids are represented by their α-carbon atoms. All the amide linkages (-CO-NH-) are represented by a line between each α-carbon atom. As shown in Figure 2, the polypeptide chain folds around the heme group causing it to reside in a nearly hydrophobic environment, leaving only a small portion of the heme exposed to the more polar solvent. Based on the X-ray structure of horse heart cytochrome c, it was reported that the amount of surface area of the heme accessible to the solvent forms only about 0.6% of the total surface area of the protein [5]. It was also reported that this solvent accessible area is a possible site for electron transfer [6].

Horse heart cytochrome c is a highly basic water soluble protein with an isoelectric point of ~10.0 [7]. It contains 19 lysine residues which are positively charged basic residues [1]. The basic arginine residues contribute two more positive charges to the protein at neutral pH. A maximum of only 13 negatively charged groups can be found, 3 aspartates, 9 glutamates, and the negative charge on the carboxyl-terminal end of the protein [1]. Counting for the +1 charge on the heme in the oxidized state, ferricytochrome c has an overall net charge of +9 at neutral pH [2]. However, it must be noted that the net charge on cytochrome c can be effectively altered in the presence of binding cations and anions. One of the important features about cytochrome c is that the negative and positive charges are distinctly segregated on its surface. The left front face contains most of the positive charge, while a very high concentration of the negative charge is located at the back left portion of the protein. The highly asymmetric distribution of charges over

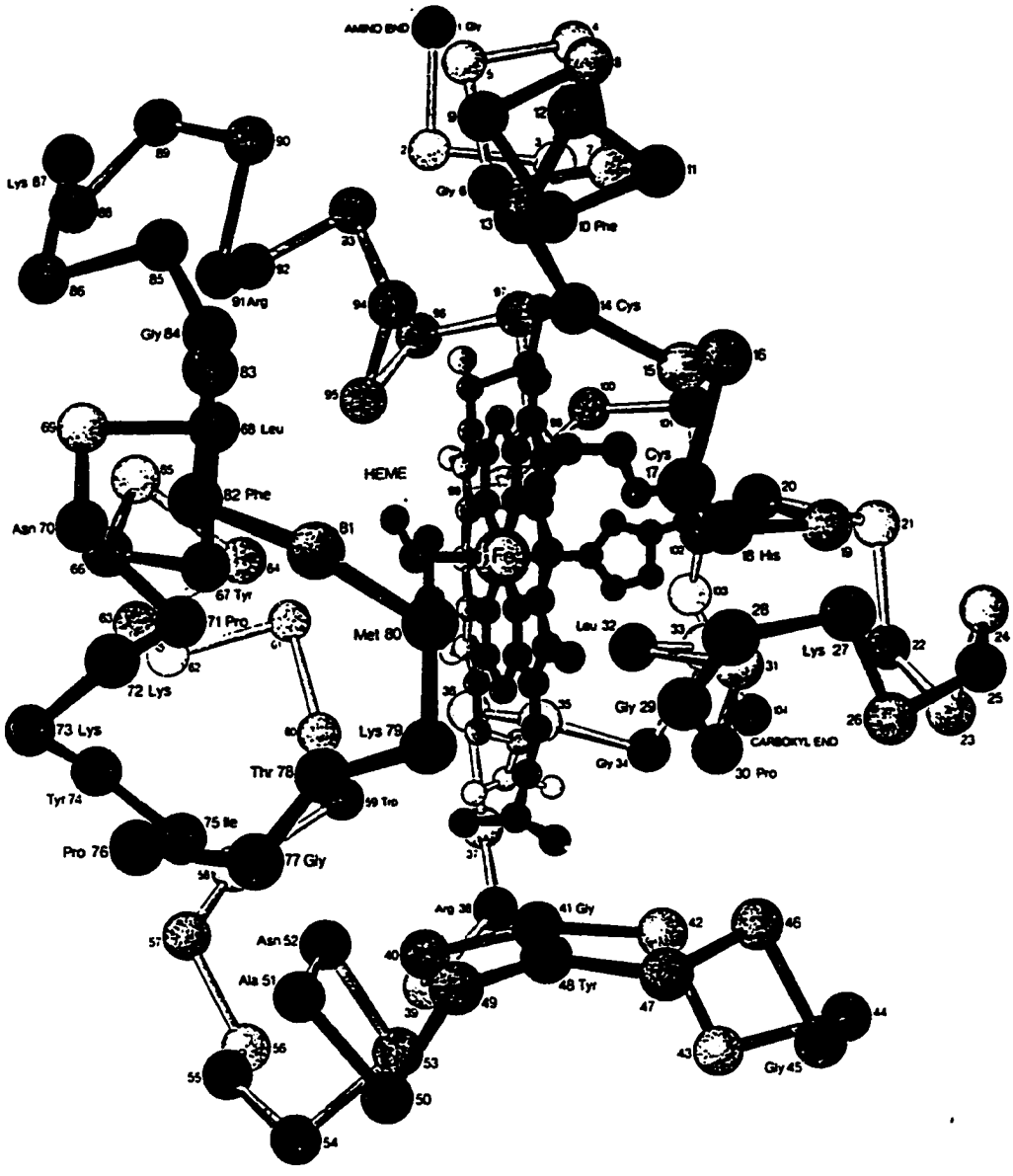

Figure 2. Molecular structure of cytochrome c. This standard front view of horse heart cytochrome c in the oxidized state, illustrates the solvent exposed heme edge in the center of the molecule facing towards the reader. Only those amino acids attached directly to the heme (Met80, His18, Cys14, Cys17) are shown, all others are represented by circles depicting α-carbon atoms of the polypeptide backbone. All amide linkages are represented by a line between each α-carbon atom (from Ref. 4).

cytochrome c surface leads to a calculated dipole moment of 312 and 300 debye for oxidized and reduced cytochrome c, respectively [8]. It was reported that the positive end of this dipole is near the β-carbon of phenylalanine 82 on the front surface [9,10]. The negative end of this dipole is near the β-carbon of the asparagine 103 on the back surface. Phenylalanine 82 is located near the solvent-accessible heme edge.

The solvent exposed heme edge is surrounded by lysines 8, 13, 27, 72, 79, 86, and 87. It has been proposed that electrostatic interactions of these charged lysine residues and corresponding dipole are important in the reactions of cytochrome c with its redox partners [9,10]. The positive end of the dipole of cytochrome c interacts with the electric fields generated by its redox partners. This is believed to guide the molecule into proper orientation for electron transfer and therefore increases the number of productive encounters.

The electrochemical properties of cytochrome c were studied as early as the 1930's [11]. However, research relating to heterogeneous electron transfer of cytochrome c did not attract too much attention until 1977 when the well defined electrochemistry of cytochrome c was observed at an indium oxide electrode [12] and at a 4,4'-bipyridine-modified gold electrode [13]. The reason for this slow development was the inherent lack of communication between cytochrome c and electrode. Such lack of communication led to slow and often negligible electron transfer reactions. Slow and irreversible heterogeneous electron transfer was attributed to the insulating properties of the protein surrounding the molecule's active site, making this site inaccessible to the electrode. Since 1977, a substantial amount of knowledge has been gathered concerning the interfacial

behavior of cytochrome c at various electrode surfaces. At the same time that indium oxide electrodes were being used, work on gold electrodes was also being carried out. Eddowes and Hill [13] were able to enhance the electron transfer reaction of cytochrome c at gold electrode, by adding 4,4'-bipyridine to the solution. Such compound was different from the electroactive organic molecules used before, called "mediators", whose formal potential was within the same range as the biological molecule of interest [14,15]. The 4,4'-bipyridine was one of the first molecules known to accelerate the electron transfer reaction of cytochrome c while being electroinactive in the potential window of the molecule being studied. Therefore, according to Hill et al., the 4,4'-bipyridine was designated a "surface promoter" for electrochemical reaction of cytochrome c.

Modifying electrode surfaces with a surface promoter opened up a new area in electrochemistry, by providing a convenient way of investigating direct heterogeneous electron transfer reactions of heme proteins. Since the initial report by Hill [13] many effective promoters have now been found which can enhance the electron transfer reactions of large biological molecules at a variety of different electrode surfaces. Taniguchi et al. [16] were the first to report the use of a pre-adsorbed promoter, bis(4-pyridyl) disulfide, which exhibited a well defined quasi-reversible electron transfer reaction of cytochrome c at a gold electrode, without the need for any promoter in solution.

Allen et al. [17] have defined the necessary structure-function relationship of various promoter molecules that facilitate the electron transfer reaction between electrode and cytochrome c. After investigating fifty-four promoters, they have proposed that, in order to be an effective promoter, a bifunctional molecule of the X~Y type is necessary.

In such a molecule, X represents a functional group which bonds to the metal surface, while Y represents a functional group positioned away from the electrode surface, out into solution. The surface active end, X, must be an electron pair donor group having either a nitrogen, phosphorous, or sulfur atom. The functional end, Y, on the other hand must be an ionic or weakly basic group capable of forming a salt bridge and/or hydrogen bond to positively charged lysine residues on cytochrome c's surface. Bifunctional promoters containing weakly basic pyridyl and aniline-like nitrogen groups were shown to be capable of hydrogen bonding to lysine residues, while promoters containing groups like carboxylate, sulfonate, and phosphate were found capable of forming salt bridge with such residues. However, in spite of the fact that 2,2'-bipyridine has two functional groups, it was reported that it exhibited no promoter effect for electron transfer reactions in cytochrome c [17-19]. Thus, it was concluded [17,19] that two functional groups must be present at each extremity of the promoter molecule. In addition, Haladjian et al. [19] suggested that the promoter molecules must have a minimal length because pyrazine which has two functional groups but is shorter than 4,4'-bipyridine, is not active as a promoter for cytochrome c electrochemistry [18,19]. The mechanisms by which surface promoters might function are still under investigation.

Parallel to the development of modified electrodes has been the rapid growth in interest towards unmodified electrode systems. In the unmodified electrode systems, there is no promoter in the cytochrome c solution, nor on the electrode surface. These systems include metal oxide electrodes [12,20-22], non-metal electrodes [23,24], and bare metal electrodes [25-27]. For these systems, certain pretreatments are required for either the

electrodes or the cytochrome c sample in order to observe the direct heterogeneous electron transfer reaction of cytochrome c.

Raman Spectroscopy of Cytochrome c

The nature of the resonance Raman effect is that only vibrational modes associated with the chromophoric group, the heme in this case, of the molecule are enhanced [28]. The consequences of this effect are very important since the remainder of the vibrational modes associated with the protein are not enhanced and therefore do not complicate the spectra. This is relevant from a physiological standpoint since the technique is both a highly selective and extremely sensitive probe of the chromophoric group, which is often the site of biological activity.

Polarization properties can also be used as a diagnostic tool in Raman spectroscopy of cytochrome c. By measuring the intensity of the scattered radiation in the parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) modes, estimates of the depolarization ratios $\rho = I_{\perp}/I_{\parallel}$ can be made. These ratios are useful in structure studies of heme proteins since the symmetry of a particular vibrational mode can be determined from them. Vibrational bands with depolarization ratios of 0.75 ± 0.1 are designated as depolarized (dp), those with depolarization ratios lower than 0.75 as polarized (p), and those with ratios greater than 0.75 as anomalously polarized (ap) [29]. Using these established depolarization ratios porphyrin ring modes can be assigned in the Raman spectrum. Totally symmetric vibrations give rise to polarized bands. Non-totally-symmetric vibrations produce depolarized Raman bands. Anti-symmetric vibrations of the porphyrin ring give rise to anomalous polarized bands.

In addition to polarization properties, resonance Raman can also provide insight into the normal modes of vibration. Resonance Raman scattering will occur when the exciting laser line is tuned into an electronic absorption band. Upon resonance with an electronic transition some of the Raman bands will be greatly enhanced. Figure 3 shows the most prominent absorption bands for cytochrome c [30]. In case of reduced cytochrome c, two absorption bands (α and β) are observed at 550 and 520 nm in the visible region, and one band (Soret) at 416 nm in the ultraviolet. For cytochrome c, the non-totally-symmetric and anti-symmetric modes are enhanced upon resonance of the laser line with either the α or β absorption bands [31,32]. On the other hand, when the laser line is in resonance with the Soret band, polarized scattering from totally symmetric modes are the most predominant vibrations in the spectrum. Therefore, the fact that only certain Raman vibrational bands are enhanced imparts a selectivity to the resonance Raman effect.

Figure 4 shows the resonance Raman spectra of cytochrome c with Soret excitation (413 nm) [30]. The two important bands are ν_4 and ν_3 which relate to the oxidation and spin states of cytochrome c. The ν_4 is an oxidation state marker band whose frequency is sensitive to the electron density in porphyrin π^*-orbitals. This ν_4 band shifts from 1360 cm^{-1} in the reduced state to 1373 cm^{-1} in the oxidized state. This has been interpreted in terms of π back donation from the Fe $e_g(d_\pi)$ orbitals to the porphyrin $e_g(\pi^*)$ orbitals [33]. Whereas the ν_4 band is only dependent on oxidation state, the ν_3 band is sensitive to changes in oxidation state, spin state, and coordination of the heme iron. Figure 5 shows the correlation chart between iron porphyrin modes and the oxidation-, spin-, and ligation-state of the heme iron [30]. For a six-coordinate low spin heme of cytochrome c, the ν_3

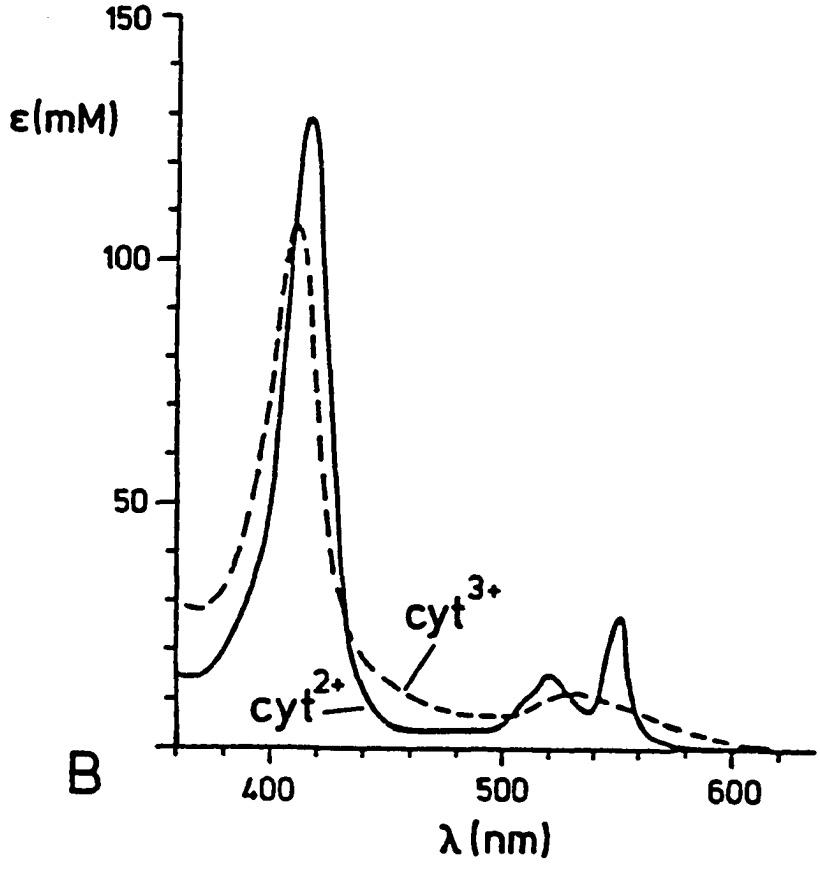

Figure 3. Adsorption spectra of cyt.c^{2+} and cyt.c^{3+} in aqueous solution (from Ref. 30).

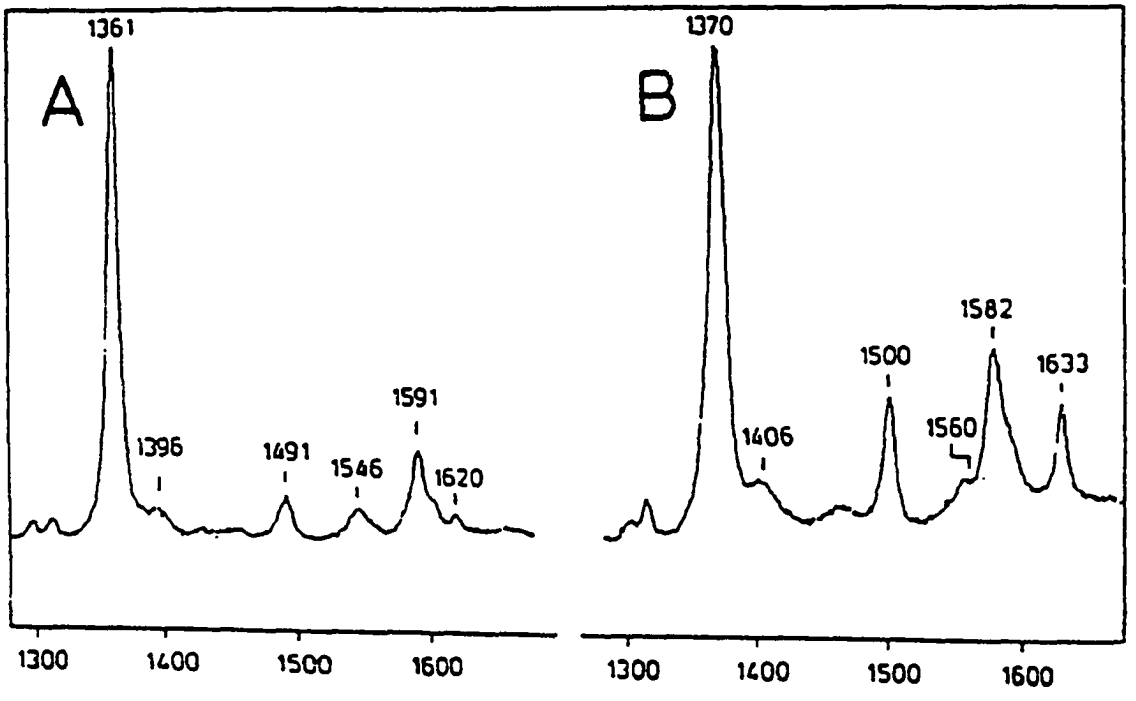

Figure 4. Resonance Raman spectra of (A) cyt.c^{2+} and (B) cyt.c^{3+} in aqueous solution obtained with 413.1 nm excitation (from Ref. 30).

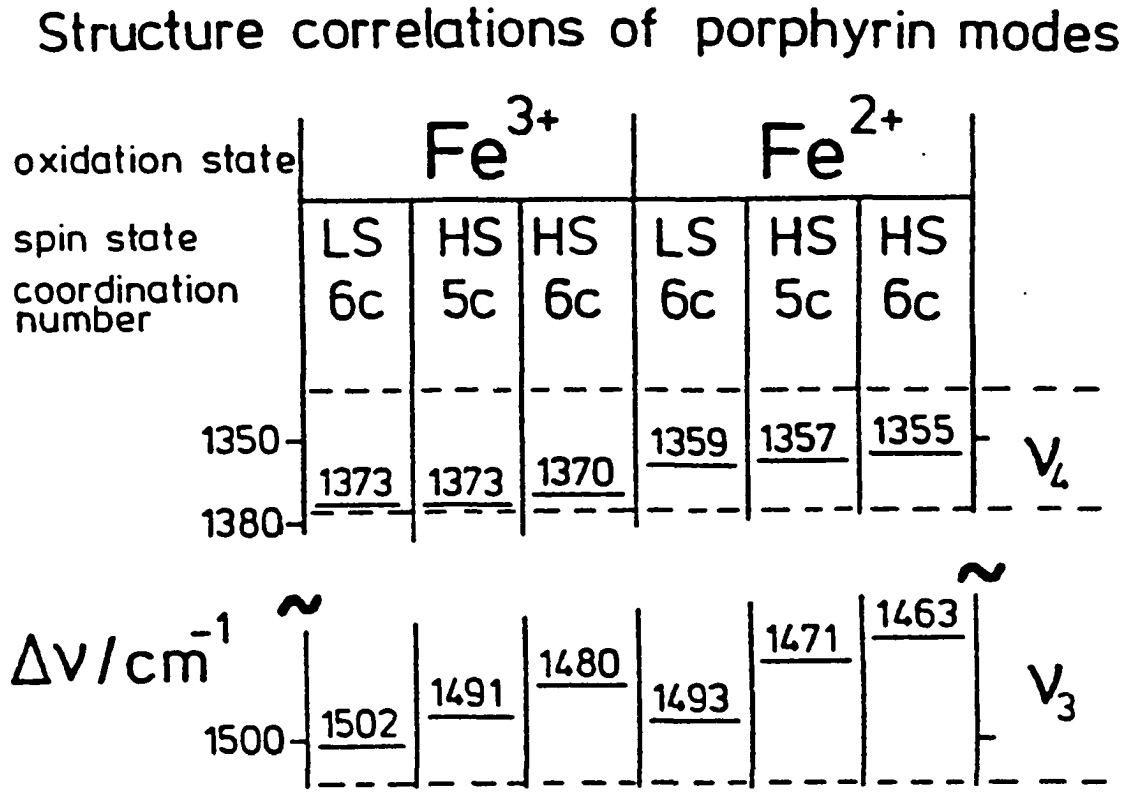

Figure 5. Correlation chart between iron porphyrin modes and the oxidation-, spin-, and ligation-state of the heme iron (from Ref. 30).

frequency is found at 1493 cm^{-1} in the reduced state and 1502 cm^{-1} in the oxidized state. In the case of a five-coordinate high-spin reduced state cytochrome c, ν_3 occurs at 1471 cm^{-1} and shifts to 1491 cm^{-1} for the oxidized state cytochrome c [33,34].

Surface-enhanced resonance Raman scattering enables one to characterize in situ the interfacial and conformational behavior of a heme protein such as cytochrome c at an electrode surface. Although both SERRS and RR spectroscopy give similar Raman spectra, there are some differences between them. In SERRS, all bands are essentially depolarized, therefore, it is not possible to use polarization measurements to determine mode symmetries of vibrational bands. Instead, bands must be correlated with previously assigned RR bands whose normal mode symmetry has been well established. The surface enhanced mechanism is dependent on the particular surface and the state or roughness of that surface. The scattering intensity profile has been shown to follow the excitation of local surface plasmon resonances [35]. Although the final mechanism of the enhancement is still debated, it is generally accepted that there are two major mechanisms called "electromagnetic" and "chemical enhancement mechanisms". In the electromagnetic enhancement theory it was proposed that the molecules adsorbed at a metal surface experience an electric field that is greater than the incident electric field [36]. In the chemical mechanism it was proposed that the molecular polarizability is increased by the perturbation from the interaction of molecules with the metal surface [37].

References

1. S. Paleus and J. B. Neilands, *Acta Chem. Scand.*, **1950**, 4, 1024.
2. E. Margolish, E. L. Smith, G. Kreil and H. Tuppy, *Nature (London)*, **1961**, 192, 1125.
3. S. Hu, I. Morris, J. P. Singh, K. M. Smith and T. G. Spiro, *J. Am. Chem. Soc.* **1993**, 115, 12446.
4. R. E. Dickerson, *Sci. Amer.*, **1972**, April, 58.
5. E. Stellwagen, *Nature*, **1978**, 275, 73.
6. K. Wuthrich, *Proc. Natl. Acad. Sci. U.S.A.*, **1969**, 63, 1071.
7. G. H. Barlow and E. Margoliash, *J. Biol. Chem.*, **1966**, 241, 1473.
8. J. D. Rush, J. Lan and W. H. Koppenol, *J. Am. Chem. Soc.*, **1987**, 109, 2679.
9. W. H. Koppenol, C. A. J. Vroonland and R. Braams, *Biochim. Biophys. Acta*, **1978**, 503, 499.
10. W. H. Koppenol and E. Margoliash, *J. Biol. Chem.*, **1982**, 257, 4426.
11. R. Brdick, *Coll. Czec. Chem. Commun.*, **1933**, 5, 112.
12. P. Yeh and T. Kuwana, *Chem, Lett.*, **1977**, 1145.
13. M. J. Eddows and H. A. O. Hill, *J. Chem. Soc. Chem. Commun.*, **1977**, 771.
14. W. R. Heineman, B. J. Norris and J. F. Goelz, *Anal. Chem.*, **1975**, 47, 79.
15. T. Kuwana and W. R. Heineman, *Acc. Chem. Res.*, **1976**, 9, 241.
16. I. Taniguchi, K. Toyosawa, H. Yamaguchi and K. Yasukouchi, *J. Chem. Soc., Chem. Commun.*, **1982**, 1032.

17. P. M. Allen, H. A. O. Hill and N. J. Walton, *J. Electroanal. Chem.*, **1984,** 178, 69.

18. M. J. Eddows and H. A. O. Hill *J. Am. Chem. Sco.*, **1979,** 101, 4461.

19. J. Haladjian, P. Bianco and R. Pilard, *Electrochim. Acta*, **1983,** 28, 1823.

20. E. E. Bancroft, H. N. Blount and F. M. Hawkridge, *Biochem. Biophys. Res. Commun.*, **1981,** 101, 1331.

21. E. E. Bancroft, H. N. Blount and F. M. Hawkridge, in *Electrochemical and Spectrochemical studies of Biological Redox Components*, K. M. Kadish, ed., American Chemical Society, Washington, DC, **1982,** 23.

22. E. E. Bancroft, H. N. Blount and F. M. Hawkridge, in *Electrochemical and Spectrochemical studies of Biological Redox Components*, K. M. Kadish, ed., American Chemical Society, Washington, DC, **1982,** 159.

23. F. B. Armstrong, H. A. O. Hill and B. N. Oliver, *J. Chem. Soc. Chem. Commun.*, **1984,** 976.

24. D. J. Arnold, K. A. Gercharlo and C. W. Anderson, *J. Electroanal. Chem.*, **1984,** 172, 379.

25. T. M. Cotton, D. Kaddi and D. Iorga, *J. Am. Chem. Soc.*, **1983,** 105, 7462.

26. B. A. Kutznetsov, *Bioelectrochem. Bioenerg.*, **1981,** 8, 681.

27. P. A. Serre, J. Haladjian and P. Bianco, *J. Electroanal. Chem.*, **1981,** 122, 327.

28. J. Tang and A. C. Albrecht, in *Raman Spectroscopy*, H. A. Szymanski ed., Plenum Press, New York, **1970,** Vol. 2, Chapt. 2.

29. T. G. Spiro and T. C. Strekas, *Proc. Natl. Acad. Sci. U.S.A.* **1972,** 69, 2622.

30. P. Hildebrandt and M. Stockburger, in *Raman Spectroscopy: Sixty Years On Vibrational Spectra and Structure*, H. D. Bist ed., Elsevier Science, Netherlands, **1989**, Vol. 17A, 443.

31. A. C. Albrecht, *J. Chem. Phys.*, **1961**, 34, 1476.

32. A. C. Albrecht and M. C. Hutley, *J. Chem. Phys.*, **1971**, 55, 4438.

33. T. G. Spiro and T. C. Strekas, *J. Am. Chem. Soc.*, **1974**, 96, 338.

34. P. M. Callahan and G. T. Babcock, *Biochemistry*, **1981**, 20, 952.

35. J. A. Creighton, C. G. Blachford and M. G. Albrecht, *J. Chem. Soc. Faraday Trans.*, **1979**, 75, 790.

36. C. A. Murray, in *Surface Enhanced Raman Scattering*, R. K. Chang ed., Plenum, New York, **1982**, 203.

37. A. Otto, in *Light Scattering in Solids*, M. Cardona ed., Springer-Verlag, New York, **1984**, Vol. 4, 289.

CHAPTER 1. REEVALUATION OF 2,2'-BIPYRIDINE AND PYRAZINE AS PROMOTERS FOR DIRECT ELECTRON TRANSFER BETWEEN CYTOCHROME C AND METAL ELECTRODES

A paper published in *Journal of Electroanalytical Chemistry*

Chengli Zhou, Jea-Ho Kim, Therese M. Cotton, Tianhong Lu and Shaojun Dong

Abstract

Results from previous electrochemical studies have indicated that 2,2'-bipyridine and pyrazine do not function as promoters for heterogeneous electron transfer between cytochrome c and metal electrodes. Their lack of activity was attributed to the improper positioning of the two functional groups in 2,2'-bipyridine and the inefficient length of pyrazine. In the present study it was determined that both 2,2'-bipyridine and pyrazine act as promoters when self-adsorbed over a sufficiently long dipping time. The promoter characteristics of these two molecules were studied and compared with those of 4,4'-bipyridine. The difference in their promoter behavior appears to result primarily from their different strengths of adsorption and not because electrodes modified with 2,2'-bipyridine or pyrazine are unsuitable for accelerating direct electron transfer reaction in cytochrome c. These results have implications regarding the mechanisms of promoter effects in electrochemical reactions of cytochrome c.

Introduction

The electrochemical reactivity of metalloproteins such as cytochrome c at bare metal electrodes, e.g. platinum [1-3], gold [3-6], nickel [7], silver [8] and mercury [4,9-10], is often highly irreversible and in some cases undetectable. The electrochemical

properties of cytochrome c were studied as early as 1933 [11]. However, research relating to direct electron transfer between metalloproteins and electrodes did not experience a rapid growth until the first clear demonstration of quasi-reversible and direct electrochemistry of cytochrome c at a 4,4'-bipyridine-modified gold electrode [5]. Since the first report of promoter activity by 4,4'-bipyridine, various promoters for direct and quasi-reversible electrochemical reactivity of metalloproteins, especially that of cytochrome c, have been identified [12-23]. Most of the promoters that have been studied are organic molecules.

After an extensive investigation of possible electron transfer promoters, Allen et al. [17] concluded that at least two functional groups are required to accelerate direct electron transfer between cytochrome c and metal electrodes, one of which binds to the surface of the metal electrode and the second of which interacts with amino acid side chains on the surface of cytochrome c. However, in spite of the fact that both 4,4'-bipyridine and 2,2'-bipyridine have two functional groups, only the former accelerated direct electron transfer between cytochrome c and metal electrodes, whereas 2,2'-bipyridine exhibited no promoter effect [12,15,17]. Thus, it was concluded [15,17] that the two functional groups must be present at each extremity of the promoter molecule. In addition, Haladjian et al. [15] suggested that the promoter molecules must have a minimal length because pyrazine which has two functional groups but is shorter than 4,4'-bipyridine, is not active as a promoter for cytochrome c electrochemistry [12,15].

In the present study, the promoter behavior of 2,2'-bipyridine and pyrazine was reexamined. Indeed, it was determined that when the two traditional methods were used

to modify metal electrodes, (i.e. a gold or silver electrode was placed into the cytochrome c solution with 2,2'-bipyridine or pyrazine, or dipped in the solution of 2,2'-bipyridine or pyrazine for few minutes and then transferred to the cytochrome c solution [12,15,16] no response was observed in the cyclic voltammetry (CV) of cytochrome c, indicating that these compounds are not effective promoters under these conditions. However, if the gold or silver electrode was dipped into a solution of 2,2'-bipyridine or pyrazine for several hours and then transferred to the cytochrome c solution, a quasi-reversible CV response was observed indicating that these molecules can function as promoters under the appropriate conditions. Their promoter characteristics were studied and compared with those of 4,4'-bipyridine. Differences in their promoter behavior appear to result primarily from their different strengths of adsorption.

Experimental

Horse heart cytochrome c (type VI, Sigma Chemical Co.) was used without further purification. 4,4'-bipyridine, 2,2'-bipyridine and pyrazine (Aldrich Chemical Co.) were the highest purity available (>99%) and were used as received. All other chemicals were reagent grade. A BAS 100 electrochemical analyzer and a conventional three-electrode electrochemical cell were used for the electrochemical measurements. The working electrode was constructed from a gold or silver rod which was sealed into glass tubing with Torr Seal (Varian). The exposed area was about 5.0 mm^2 for the gold electrode and 3.0 mm^2 for the Ag electrode. A Pt wire was used as the auxiliary electrode. A saturated calomel electrode (SCE) served as a reference electrode and all the potentials are reported with respect to the SCE.

The modified electrodes were prepared according to the following procedure. The working electrode was sequentially polished with 5, 0.3, 0.05 μm alumina/water slurries until a shiny, mirrorlike finish was obtained. It was then sonicated in distilled water and washed thoroughly with distilled water. For the SERS measurements on Ag, the electrode was roughened in a 0.1 M Na_2SO_4 by an oxidation reduction cycle (ORC). This consisted of a double-potential step from -500 mV to +500 mV, where 25 mC/cm^2 charge was allowed to pass, and the electrode was stepped back to -500 mV to reduce the Ag^+. The ORC was performed in the dark in order to get better roughened surfaces. For SERS measurements on Au, the electrode was cleaned prior to ORC by cathodization at -2.2 V for 10 seconds. It was then roughened by a computer controlled ORC procedure [24]. The electrode potential was varied from -300 mV to +1300 mV for 25 cycles in a 0.1 M KCl solution.

Following the polishing, the surface modification was performed by dipping the electrode into a 1 mM solution of the promoter for a defined time period, after which the electrode was rinsed twice with distilled water. Next, the promoter-modified working electrode was mounted in the electrochemical cell containing 0.38 mM cytochrome c solution, 0.025 M phosphate buffer at pH 7 and 0.1 M sodium perchlorate as a supporting electrolyte.

Surface enhanced Raman Scattering (SERS) spectra were acquired from the sample adsorbed on a silver sol. The silver sol was prepared according to a literature procedure [25]. One liter of 1 mM silver nitrate solution was degassed by heating to the boiling point. Next, 20 ml of 3.4 mM sodium citrate solution was added dropwise to the silver

nitrate solution with vigorous stirring. The mixture was maintained at boiling point for one hour and the final volume was adjusted to 1 liter with distilled water. The samples were prepared by mixing one volume of 1 mM aqueous solution of the promoter with an equal volume of silver sol. In order to compare the relative adsorption strengths of two promoters, one volume of an aqueous solution containing a mixture of the two promoters, each at 0.5 mM concentration, was mixed with one volume of silver sol. The samples were transferred to 5 mm glass tubes for Raman measurements. For the SERS experiments at 2,2'-bipyridine-modified electrodes (Ag and Au), the roughened electrodes were dipped into a 1 mM 2,2'-bipyridine solution for 30 minutes, after which the electrodes were rinsed with distilled water. Then the 2,2'-bipyridine-modified electrodes were transferred to the electrolyte solution containing 0.025 M phosphate buffer at pH 7 and 0.1 M sodium perchlorate for SERS measurements.

The Raman instrumentation used in these experiments has been described previously [26]. SERS spectra were acquired in the backscattering geometry. The 488.0 nm or 514.5 nm (ca. 30 mW) of a Coherent Innova 90-5 Ar^+ laser was used as the excitation source for SERS measurements on Ag, and the 647.1 nm line of a Coherent Innova 100 Kr^+ was used for SERS measurements on Au. The resolution of the Raman instrument is ca. 2 cm^{-1} at the excitation wavelengths used here. Indene was used to calibrate the Raman frequencies.

Results

In previous studies [12,15,16], two methods were used to modify gold electrodes for cytochrome c electrochemistry. In one method, the electrode was placed into the

solution of cytochrome c with 1 mM of 2,2'-bipyridine or pyrazine. In the second method, the electrode was dipped into a 1 mM solution of 2,2'-bipyridine or pyrazine for a few minutes and then transferred to a cytochrome c solution. When these methods were used to modify a gold electrode in the present study, no response was observed in the cyclic voltammetry measurements on cytochrome c confirming that 2,2'-bipyridine and pyrazine are inactive under these conditions. This result is consistent with that reported by Eddowes and Hill [12]. However, it was found that when the gold electrode was dipped in 1 mM 2,2'-bipyridine or pyrazine solution for more than 30 minutes, then rinsed with distilled water and placed in the cytochrome c solution, protein reduction was observed near 0.01 V, the formal potential of cytochrome c. Figure 1A and 1B show cyclic voltammograms of cytochrome c at 2,2'-bipyridine- and pyrazine-modified electrodes. It can be seen that for relatively short dipping periods (Curves a and b), the curves are sigmoidal in shape. Possible reasons for this shape will be discussed below. With dipping times of 6 hours or greater, distinct peaks were observed in the CV response and the peak separation decreased as the dipping time was increased. Similar results were also observed using silver electrodes. Figure 1D shows the CV response of cytochrome c at a silver electrode that was exposed to pyrazine for increasing time periods. A comparison of Figure 1B and 1D shows that pyrazine is a more effective promoter with Au electrodes as compared with Ag. The reduction current is less and the peak separation is greater for pyrazine-modified Ag electrodes even after 12 h of exposure to the pyrazine solution.

The peak separation (ΔE_p) in the CV response for cytochrome c obtained from

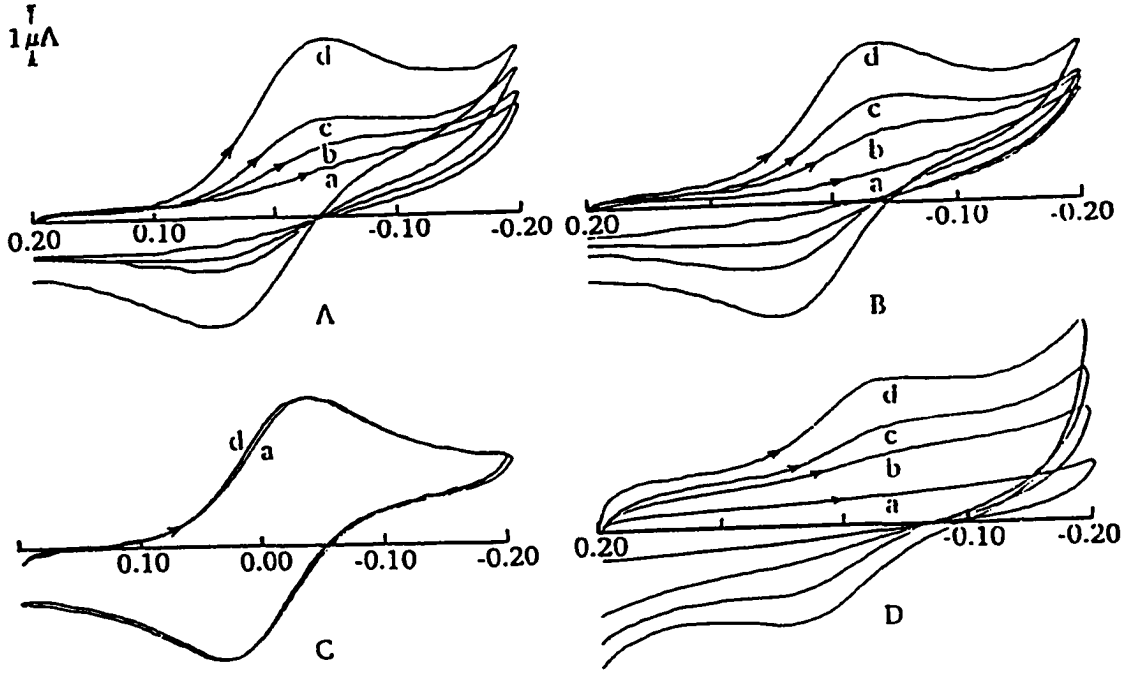

Figure 1. Cyclic voltammograms of 0.38 mM cytochrome c at a (A) 2,2'-bipyridine-modified gold electrode; (B) pyrazine-modified gold electrode; (C) 4,4'-bipyridine-modified gold electrode and (D) pyrazine-modified silver electrode in phosphate buffer solution (pH = 6.97) with 0.1 M $NaClO_4$. The dipping times were as follows: (a) 2 min.; (b) 30 min.; (c) 6 h.; (d) 12 h. The scan rate was 50 mV/s and the initial potential was +0.2 V.

electrodes which were dipped for 12 h in 4,4'-bipyridine solution (Figure 1C) is about 60 mV. The ΔE_p for 2,2'-bipyridine- and pyrazine-modified electrodes is approximately 80 mV at a scan rate of 50 mV/s, which is nearly the same as that observed for cytochrome c at bis(4-pyridyl) disulfide- [13] or purine-modified [16] electrodes and only slightly greater than that for a fully reversible one-electron redox reaction. The midpoint between the cathodic and anodic peak potentials is near 0.01 V which is in good agreement with the formal potential of cytochrome c [12,13]. The ratio of the anodic to cathodic peak current is approximately unity.

Curve a in Figure 2 illustrates the relationship between the peak separation in the cytochrome c cyclic voltammograms for exposure of the electrodes to 2,2'-bipyridine for time periods ranging between 6 and 14 h (i.e. conditions under which distinct peaks are observed in the CV response). It can be seen that initially the peak separation decreases rapidly with dipping time and reaches a stable value after about 10 h. The same behavior was observed for pyrazine-modified electrodes. In contrast with the above results, the performance of the 4,4'-bipyridine-modified electrode was optimal after exposing the electrode to the solution for only a few minutes and no further change resulted with increased dipping time (Figure 2, curve b).

Figure 3 shows the CV response for cytochrome c at a 2,2'-bipyridine-modified gold electrode at different scan rates. A plot of the peak current (corrected for the background current) for both the cathodic and anodic peaks shows a linear increase as a function of the square root of the scan rate (Figure 4). The same behavior was observed for pyrazine-modified electrodes, demonstrating that the electrochemical reaction rate of

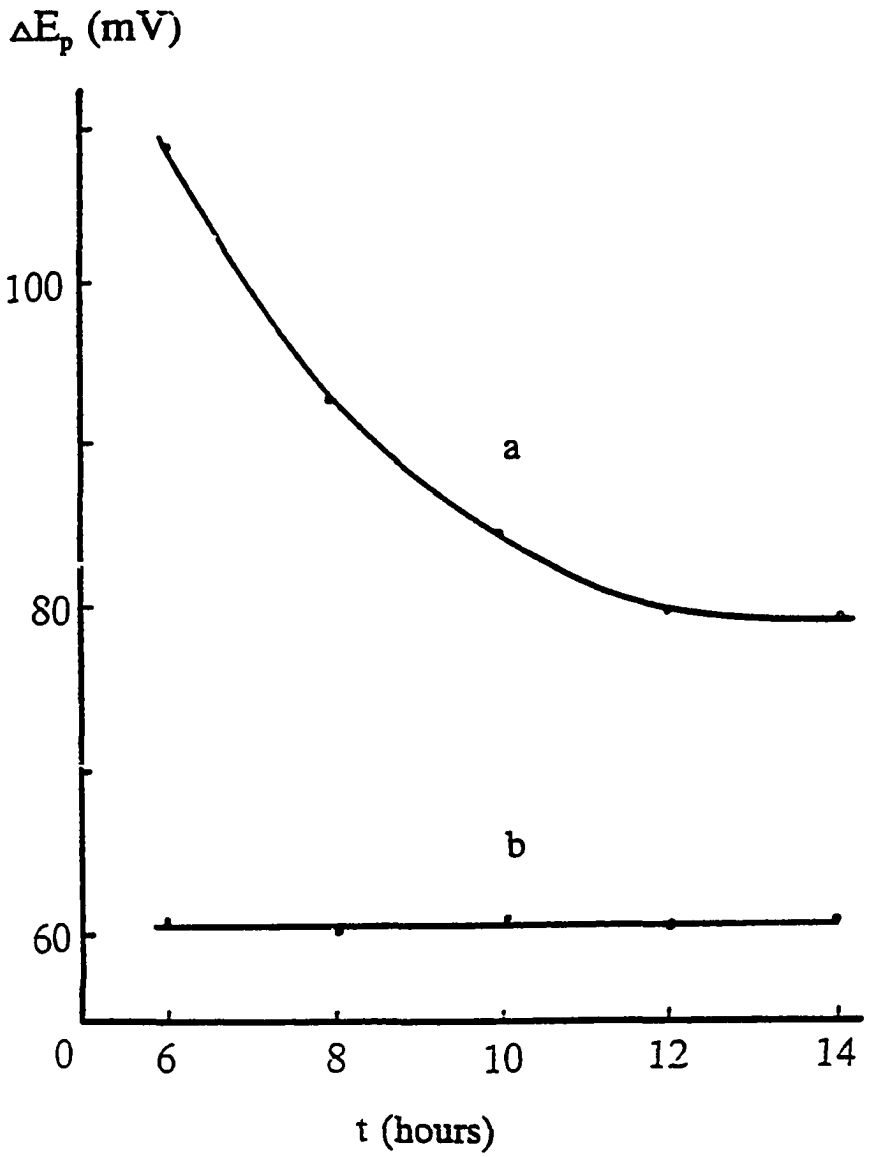

Figure 2. Dependence of the peak separation in the cyclic voltammograms of cytochrome c on the dipping time of the modification procedure: (a) 2,2'-bipyridine-modified gold electrode, (b) 4,4'-bipyridine-modified gold electrode.

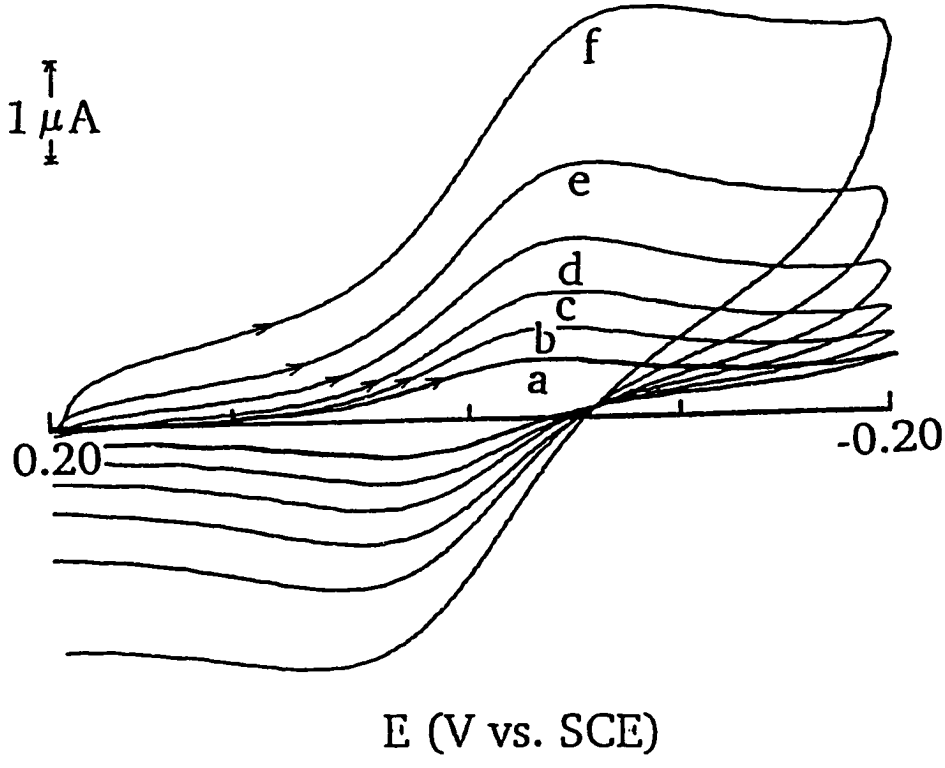

Figure 3. Cyclic voltammograms of 0.38 mM cytochrome c at a 2,2'-bipyridine-modified gold electrode in phosphate buffer solution (pH = 7.0) with 0.1 M $NaClO_4$ as the electrolyte at the following scan rates: (a) 10; (b) 25; (c) 50; (d) 100; (e) 200; (f) 500 mV/s. The electrode was dipping in the 2,2'-bipyridine solution for 12 h. The initial potential was +0.2 V.

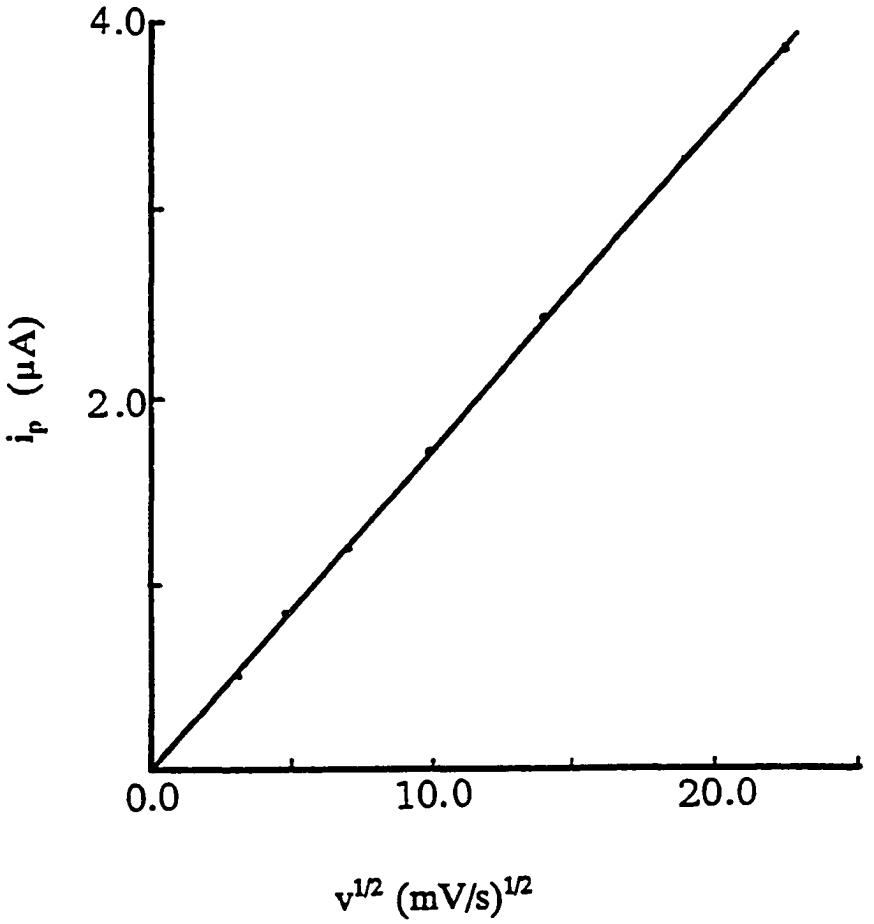

Figure 4. Scan rate dependence of the peak current for cytochrome c reduction at a 2,2'-bipyridine-modified gold electrode.

cytochrome c at 2,2'-bipyridine- or pyrazine-modified gold electrode is diffusion-controlled. In addition, 2,2'-bipyridine and pyrazine have no electrochemical response in the potential range from -0.2 V to +0.2 V. Therefore, all of the above characteristics suggest a quasi-reversible, direct electron transfer reaction between cytochrome c and 2,2'-bipyridine- or pyrazine-modified gold electrodes that were prepared using a 12 h dipping period. Thus, 2,2'-bipyridine and pyrazine are effective promoters when adsorbed on the electrode surface.

The lifetime of the 2,2'-bipyridine- or pyrazine-modified electrode is different from that of a 4,4'-bipyridine-modified electrode. For example, after 25 continuous cycles the CV response of cytochrome c at 4,4'-bipyridine-modified electrode is changed only slightly (Figure 5C). However, the stability of the 2,2'-bipyridine- or pyrazine-modified electrode is much less than that of the 4,4'-bipyridine-modified electrode. After 25 continuous cycles, the redox peaks for cytochrome c at electrodes modified with the former compounds are barely observed (Figures 5A and 5B, curve f). Moreover, if the 2,2'-bipyridine- or pyrazine-modified electrodes are allowed to remain in contact with cytochrome c solution for 5-10 min. without scanning, the redox peaks also disappear.

In the above experiments, no promoter was present in the cytochrome c solution. If, after modification of the electrode was complete, 1 mM 2,2'-bipyridine was added to the cytochrome c solution before electrochemical measurements were undertaken, the performance of the electrode decreased only slightly after 25 continuous cycles (Figure 5D, curve f). However, when 1 mM pyrazine was added to the cytochrome c solution, the peak currents decreased at the same rate as in the absence of pyrazine.

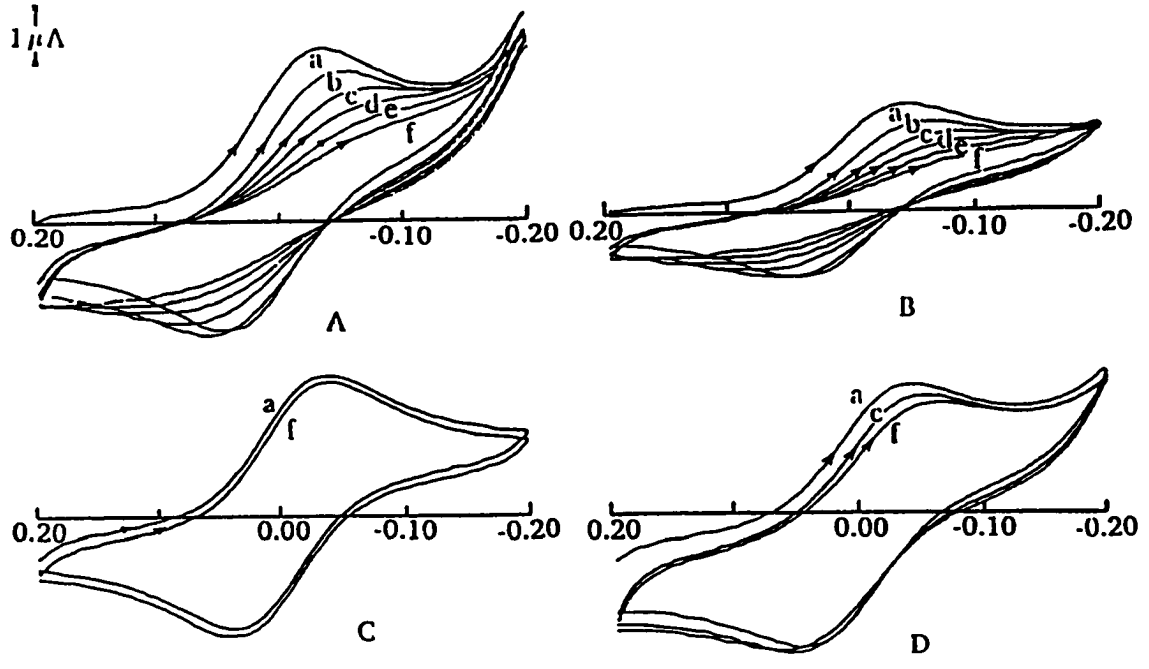

Figure 5. Cyclic voltammograms of 0.38 mM cytochrome c solution at (A) 2,2'-bipyridine-, (B) pyrazine-, and (C) 4,4'-bipyridine-modified gold electrodes as a function of the number of scans: (a) 1st; (b) 5th; (c) 10th; (d) 15th; (e) 20th; (f) 25th cycle. In Figure (D), the conditions were the same as for (A) except that 1 mM 2,2'-bipyridine was added to the cytochrome c solution. The scan rate was 50 mV/s and the initial potential was +0.2 V.

The different promoter behavior of 2,2'-bipyridine, pyrazine and 4,4'-bipyridine described above appears to be directly related to the different adsorption strengths of these promoters on the gold or silver surfaces. This was determined from surface-enhanced Raman scattering (SERS) spectroscopy, as will be described below. Based upon the spectroscopic results, the relative strengths of adsorption are as follows: 4,4'-bipyridine > 2,2'-bipyridine > pyrazine.

SERS provides a powerful method for comparing the adsorption behavior of a broad range of molecules on metal surfaces [27,28]. However, the observation of SERS signals requires that the surface is roughened by one of several procedures. Most commonly, an oxidation reduction cycle is used. The 2,2'-bipyridine-modified Au and Ag electrodes were examined by SERS. Two types of experiments were performed. First, the relative adsorption strength of the modifiers was compared by adding 1 ml each of 1 mM solution of 2,2'-bipyridine and 4,4'-bipyridine to 2 ml of a silver sol. The resulting SERS spectrum was that of 4,4'-bipyridine alone, showing that the adsorption ability of 4,4'-bipyridine on silver surface is stronger than that of 2,2'-bipyridine. Similarly, it was found that the adsorption ability of 2,2'-bipyridine is stronger than that of pyrazine. Finally, the same procedure was used for a mixture of pyrazine and cytochrome c, and only the spectrum of cytochrome c was observed, indicating that cytochrome c is more strongly adsorbed than pyrazine.

In the second type of experiment, SERS spectra were obtained on Ag and Au electrodes. Figure 6 shows that the 2,2'-bipyridine-modified Au and Ag electrodes give rise to the same SERS spectra in the presence of the electrolyte solution used for the

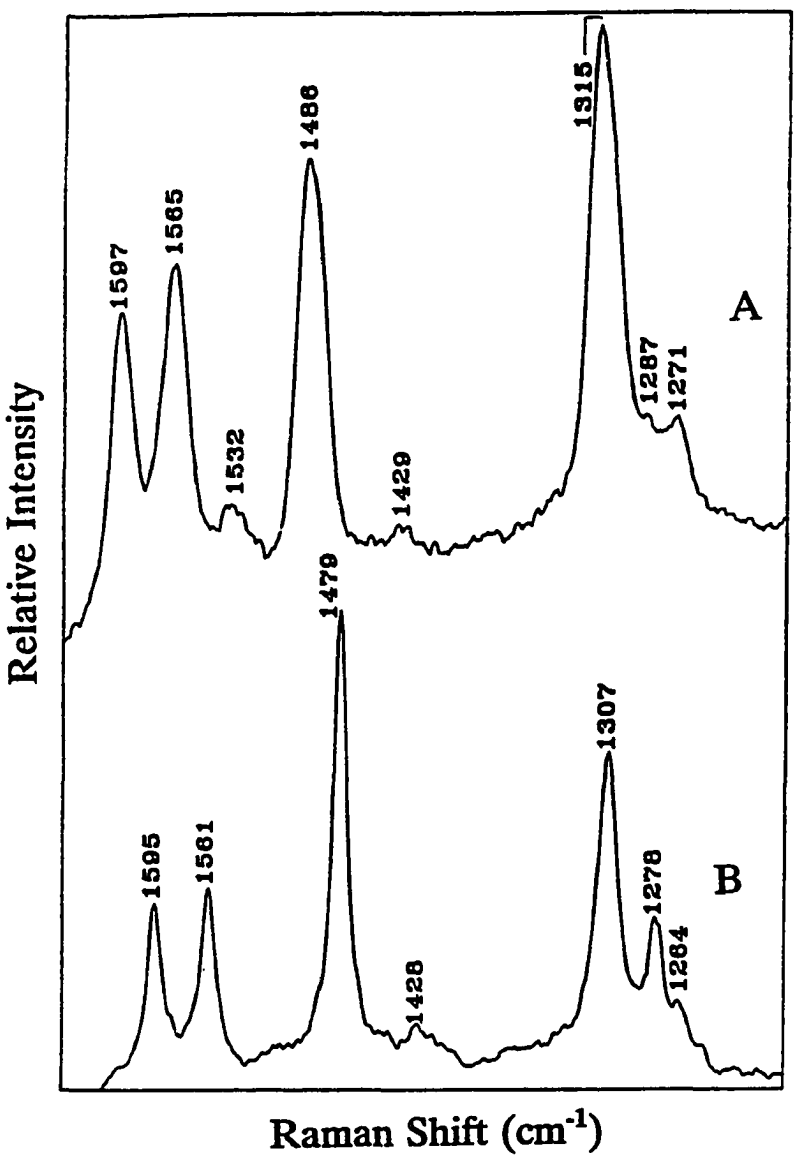

Figure 6. Surface-enhanced Raman scattering (SERS) spectra of 2,2'-bipyridine modified (A) Au and (B) Ag electrodes in 0.1 M $NaClO_4$ containing 25 mM phosphate buffer pH 7. The electrode potential was -200 mV. SERS spectra were recorded using 647.1 nm excitation (Au) and 514.5 nm excitation (Ag). The laser power was 100 mW and the spectra are composed of 25 scans. The integration time was 1 second per scan.

cytochrome c electrochemistry. An analysis of the spectra showed that the 2,2'-bipyridine is adsorbed in the cis form, as was observed in previous investigations [29,30].

Discussion

The results described above demonstrate that when a self-adsorption procedure is used with a sufficiently long dipping time, both 2,2'-bipyridine and pyrazine can function as electron transfer promoters and accelerate the electrochemical reactivity of cytochrome c. Thus, it appears that an effective promoter does not require a structure with two functional groups at each extremity of the molecule or a minimal length as suggested by Allen et al. [17] and Haladjian et al. [15]. In the case of 2,2'-bipyridine molecule the two nitrogen atoms are located on the same side of the molecule (cis isomer) when coordinated to metal ions. The SERS spectra verify that this structure is also present on Ag and Au electrode surfaces. Under these conditions it is not possible to have one nitrogen bound to the electrode surface while the other is attached to the cytochrome c molecule. To observe the promoter effect, a much longer dipping time is required for 2,2'-bipyridine and pyrazine, as compared to 4,4'-bipyridine. This requirement appears to be related to their different adsorption strengths, as demonstrated by SERS.

The adsorption strength affects not only the dipping time, but also the lifetime of the modified electrodes. Because the adsorption strength of 2,2'-bipyridine or pyrazine on gold or silver surface is weaker than that of 4,4'-bipyridine, the performance of electrodes modified with the former two compounds declines rapidly with repeated cycling of the electrode potential whereas the performance of 4,4'-bipyridine-modified electrode is stable. Competitive adsorption between the promoter and cytochrome c is also an

important factor with respect to the lifetime of the modified electrodes. The electrochemical stability of the modified electrode in cytochrome c solution is greater when 2,2'-bipyridine is also present in solution indicating that the adsorption ability of 2,2'-bipyridine on gold surface appears comparable to that of cytochrome c. In contrast, the presence of pyrazine in the cytochrome c solution does not affect the lifetime of the pyrazine-modified electrode. This is because the adsorption strength of pyrazine is much weaker than that of cytochrome c and the adsorbed pyrazine molecules are replaced with cytochrome c molecules even in the presence of pyrazine. Once cytochrome c is adsorbed directly on the surfaces of bare metal electrodes it can undergo conformational changes which lead to a change in its redox potential and the loss of electron transfer activity [31]. The adsorbed protein can block electron transfer between the electrode and cytochrome c in solution.

The shape of the cytochrome c cyclic voltammograms at 2,2'-bipyridine-modified electrodes is reminiscent of the sigmoidal shape that is characteristic of radial diffusion. As pointed out by Armstrong et al. [32] such curves can be interpreted in terms of a microscopic model of the electron transfer reaction of cytochrome c at graphite electrodes. Similarly, the electrochemical response of two other proteins, plastocyanin and ferredoxin, was successfully analyzed in terms of this model [33]. The results reported herein support the possibility that microscopic regions of the electrode are active during the initial stages of electrode modification with weakly adsorbed species such as 2,2'-bipyridine. Following long term exposure to the modifiers, the response is typical of a linear diffusion process and peak shaped voltammograms are observed.

From the above results it appears that a major requirement for an effective promoter is that it adsorb strongly to the metal surface. Consequently, a correct evaluation of the effect of molecular structure on the promoter ability requires a consideration of the adsorption strength of the molecule. The fact that a promoter effect is not observed for 2,2'-bipyridine following a dipping time of only few minutes does not indicate that the structure of these molecules is not appropriate for promoter activity. It is important that other immobilization methods be attempted, such as self-adsorption with varying dipping times. A similar result was reported by Bartlett and Farington [23] who found that 5-carboxyindole did not show a promoter effect when it was added to a cytochrome c solution. However, when the compound was coated on the electrode surface by electropolymerization a promoter effect was observed. From this point of view, adsorption over a prolonged dipping time for the modification procedure may be better than the method commonly used to evaluate the effect of the structure on promoter effects. This approach should minimize differences caused by variations in the adsorption strength of the molecules. Further experiments are underway to examine a series of potential new promoters and to explore the nature of the promoter mechanisms.

Acknowledgments

The authors are grateful for the financial support of the National Institute of Health (GM 35108 TMC) and the National Nature Science Foundation of China for this research.

References

1. T. Kono and S. Nakamura, *Bull. Agr. Chem. Soc. Jpn.*, **1958**, 22, 399.
2. S. R. Betso, M. H. Klapper and L. B. Anderson, *J. Am. Chem. Soc.*, **1972**, 94, 8197.
3. M. R. Tarasevich and V. A. Bogdanovskaya, *Bioelectrochem. Bioenerg.*, **1976**, 3, 589.
4. W. R. Heineman, B. J. Norris and J. F. Goelz, *Anal. Chem.*, **1975**, 47, 79.
5. M. J. Eddowes and H. A. O. Hill, *J. Chem. Soc. Chem. Comm.*, **1977**, 771.
6. N. S. Lewis and M. S. Wrighton, *Science*, **1981**, 211, 944.
7. B. A. Kuznetsov, N. M. Mestechkina, M. V. Izotov, I. I. Karuzina, A. V. Karyakin and A. I. Archakov, *Biochemistry*, **1979**, 44, 1234.
8. T. M. Cotton, S. G. Schultz and R. P. Van Duyne, *J. Am. Chem. Soc.*, **1980**, 102, 7960.
9. L. Griggio and S. Pinamonti, *Atti. Ist. Veneto Sci. Lett. Arti. Cl. Sci. Mat. Nat.*, **1965-1966**, 124, 15.
10. F. Scheller, M. Janchen, G. Etzold and H. Will, *Bioelectrochem. Bioenerg.*, **1974**, 1, 478.
11. R. Brdicka, *Coll. Czec. Chem. Commun.*, **1933**, 5, 112.
12. M. J. Eddowes and H. A. O. Hill, *J. Am. Chem. Sco.*, **1979**, 101, 4461.
13. I. Taniguchi, K. Toyosawa, H. Yamaguchi and K. Yasukouchi, *J. Electroanal. Chem.*, **1982**, 140, 187.

14. V. J. Razumas, A. S. Samalius and J. J. Kulys, *J. Electroanal. Chem.*, **1984**, 164, 195.

15. J. Haladjian, P. Bianco and R. Pilard, *Electrochim. Acta*, **1983**, 28, 1823.

16. I. Taniguchi, M. Iseki, K. Toyosawa, H. Yamaguchi and K. Yasukouchi, *J. Electroanal. Chem.*, **1984**, 164, 385.

17. P. M. Allen, H. A. O. Hill and N. J. Walton, *J. Electroanal. Chem.*, **1984**, 178, 69.

18. K. Di Gleria, H. A. O. Hill, V. J. Lowe and D. J. Page, *J. Electroanal. Chem.*, **1986**, 213, 333.

19. T. Yokota, K. Itoh and A. Fujishima, *J. Electroanal. Chem.*, **1987**, 216, 289.

20. H. A. O. Hill, D. J. Page and N. J. Walton, *J. Electroanal. Chem.*, **1987**, 217, 141.

21. F. A. Armstrong and K. J. Brown, *J. Electroanal. Chem.*, **1987**, 219, 319.

22. M. Shibata and N. Furuya, *J. Electroanal. Chem.* **1988**, 250, 201.

23. P. N. Bartlett and J. Farington, *J. Electroanal. Chem.*, **1989**, 261, 471.

24. P. Gao, D. Gosztola, L-W. H. Leung and M. J. Weaver, *J. Electroanal. Chem.*, **1987**, 211, 233.

25. R. S. Sheng, L. Zhu and M. D. Morris, *Anal. Chem.*, **1986**, 58, 1116.

26. T. Lu and T. M. Cotton, *J. Phys. Chem.*, **1987**, 91, 5978.

27. S. M. Heard, F. Grieser, C. G. Barraclough and J. V. Sanders, *J. Phys. Chem.*, **1985**, 89, 389.

28. Y. Ikezawa, H. Saito, K. Matsui and G. Toda, *Surf. Sci.*, **1986**, 176, 603.

29. M. Kim and K. Itoh, *Chem. Lett.*, **1984**, 357.

30. C. M. R. Mahoney and M. W. Howard, *Langmuir* **1985**, 1, 273

31. F. A. Armstrong, H. A. O. Hill and N. J. Walton, *Acc. Chem. Res.*, **1988**, 21, 407.

32. F. A. Armstrong, A. M. Bond, H. A. O. Hill, I. S. M. Psalti and C. G. Zoski, *J. Phys. Chem.*, **1989**, 93, 6485.

33. A. M. Bond, H. A. O. Hill, D. J. Page, I. S. M. Psalti and N. J. Walton, *Eur. J. Biochem.*, **1990**, 191, 737.

CHAPTER 2. DIRECT ELECTROCHEMISTRY OF CYTOCHROME C AT GOLD ELECTRODES MODIFIED WITH ORGANIC COMPOUNDS CONTAINING ONE FUNCTIONAL GROUP

Introduction

Cytochrome c does not undergo a fast and direct electron transfer at metal electrodes despite the fact that it is readily oxidized or reduced by chemical reagents and in the living organisms [1,2]. Thus, research relating to direct electron transfer between cytochrome c and a metal electrode did not experience a rapid growth until the first clear demonstration of quasi-reversible and direct electrochemical reaction of cytochrome c at the 4,4'-bipyridine-modified gold electrode by Hill and coworkers [3]. 4,4'-bipyridine was termed a "promoter" of the electron transfer process because it does not undergo electrochemical reactions within the potential range used to reduce or oxidize cytochrome c.

Following the initial observation of Hill and coworkers, an extensive research effort has been devoted to the study of new promoters and the mechanism of the heterogeneous electron transfer process between cytochrome c and the promoter-modified metal electrode. To date, most of the effective promoters that have been studied are organic compounds, such as 1,2-bis-(4-pyridyl) ethylene [3], bis(4-pyridyl) disulfide [4] and purine [5] etc. After the study of more than 50 organic promoters, Hill and his coworkers concluded [6,7] that a promoter should be bifunctional.

In the previous chapter [8], it was reported that 2,2'-bipyridine is an effective promoter. It was found that a major requirement for an effective promoter is that it adsorbed strongly at the metal surface. In addition, surface-enhanced Raman spectroscopy studies showed that 2,2'-bipyridine adsorbed on a gold surface is in the *cis* form, and thus it was inferred that compounds with only one functional group may also be effective promoters.

In this study, several compounds with only one functional group, including carbazole, pyridine and thiophene, were examined as potential promoters. All three were found to behave as promoters, but their effectiveness is somewhat different because of variations in their adsorption strength at the gold surface.

Experimental

Horse heart cytochrome c (type VI, Sigma Chemical Co.) was used without further purification. Carbazole, pyridine and thiophene (Aldrich Chemical Co.) were the highest purity available (>99%) and were used as received. All other chemicals were reagent grade.

A BAS 100 electrochemical analyzer and a conventional three-electrode electrochemical cell were used for the electrochemical measurements. The working electrode was constructed from a gold rod which was sealed into glass tubing with Torr seal (Varian). The exposed area was approximately 8 mm^2. A Pt wire was used as the auxiliary electrode. A saturated calomel electrode (SCE) served as the reference electrode and all the potentials are reported with respect to the SCE.

The film transfer method [5,6] was used to evaluate the ability of carbazole,

pyridine and thiophene to function as promoters. In this method, the modified electrodes were prepared by the following procedure. The working electrode was sequentially polished with 5, 0.3, 0.05 μm alumina/water slurries until a shiny, mirrorlike finish was obtained. The electrode was then sonicated in deionized water and washed thoroughly with deionized water. Surface modification of the working electrode was carried out by dipping the freshly polished working electrode into 1 mM pyridine or thiophene solution, or into a saturated solution of carbazole for a defined time period, followed by rinsing twice with deionized water.

The electrochemical studies of cytochrome c were carried out at the modified electrode in 0.38 mM cytochrome c solution with 0.025 M phosphate buffer at pH 7.0 and 0.1 M sodium perchlorate. Oxygen was purged from solution by bubbling with nitrogen for 10 minutes prior to the electrochemical measurement. The scan rate was usually 50 mV/s and the initial potential was +0.2 V. The potential range of scanning was from -0.2 V to +0.2 V.

After surface modification of the working electrode, the surface of the modified electrode was dried in air and then X-ray photoelectron spectroscopy (XPS) measurements were carried out at the normal operating temperature (315 K) using a ESCALAB-MK II spectrometer with the monochromatic Al K_α radiation. The spectra calibrations were made with respect to the C(1s) spectral line at 284.6 eV.

Results

Cytochrome c did not undergo reversible electron transfer at a freshly polished gold electrode, and no response was observed in its cyclic voltammogram (CV). After

modification of a freshly polished gold electrode with thiophene, it was found that its open-circuit potential was about 150 mV more negative than that for the unmodified electrode. In addition, a well-defined pair of redox peaks in the CV of cytochrome c was observed at the thiophene-modified gold electrode after a 60 minute dipping time in the modification procedure (Figure 1). This current is entirely due to cytochrome c because thiophene exhibits no electrochemical response in the potential range from -0.2 V to +0.2 V. The difference ΔE_p between the cathodic and anodic peak potential is about 72 mV, which is only slightly larger than that for a fully reversible one-electron transfer reaction. According to Nicholson's method [9], the heterogeneous electron transfer rate constant, k_s, was determined to be 4.95×10^{-3} cm/s. The midpoint between the cathodic and anodic peak potentials was approximately +0.01 V, which is close to the formal potential of cytochrome c [10]. The ratio of the anodic to cathodic peak current is approximately unity. The cathodic and anodic peak currents are proportional to the square root of the scan rate in the range 10-500 mV/s, indicating that the reaction is diffusion controlled. All the characteristics mentioned above demonstrate that a quasi-reversible, direct electrochemical reaction of cytochrome c occurs at the thiophene-modified gold electrode.

The performance of the thiophene-modified gold electrode is very stable, with the peak currents showing almost no change after 100 continuous cycles. Even after several days, the CV response was nearly the same as that observed initially. Therefore, it can be concluded that thiophene is an excellent promoter.

It was found that the performance of the thiophene-modified gold electrode is dependent on the dipping time. Figure 2 shows dependence of the ΔE_p in the CV of

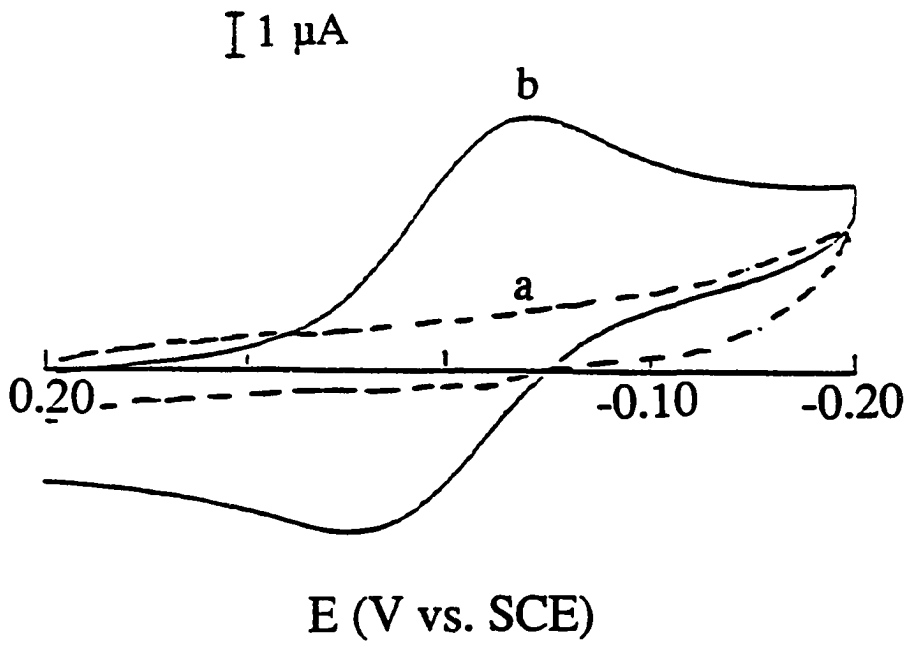

Figure 1. Cyclic voltammograms of 0.38 mM cytochrome c at (a) a freshly polished gold electrode and (b) a thiophene-modified gold electrode in phosphate buffer solution (pH 7.0) with 0.1 M $NaClO_4$. The scan rate was 50 mV/s and the initial potential was +0.2 V.

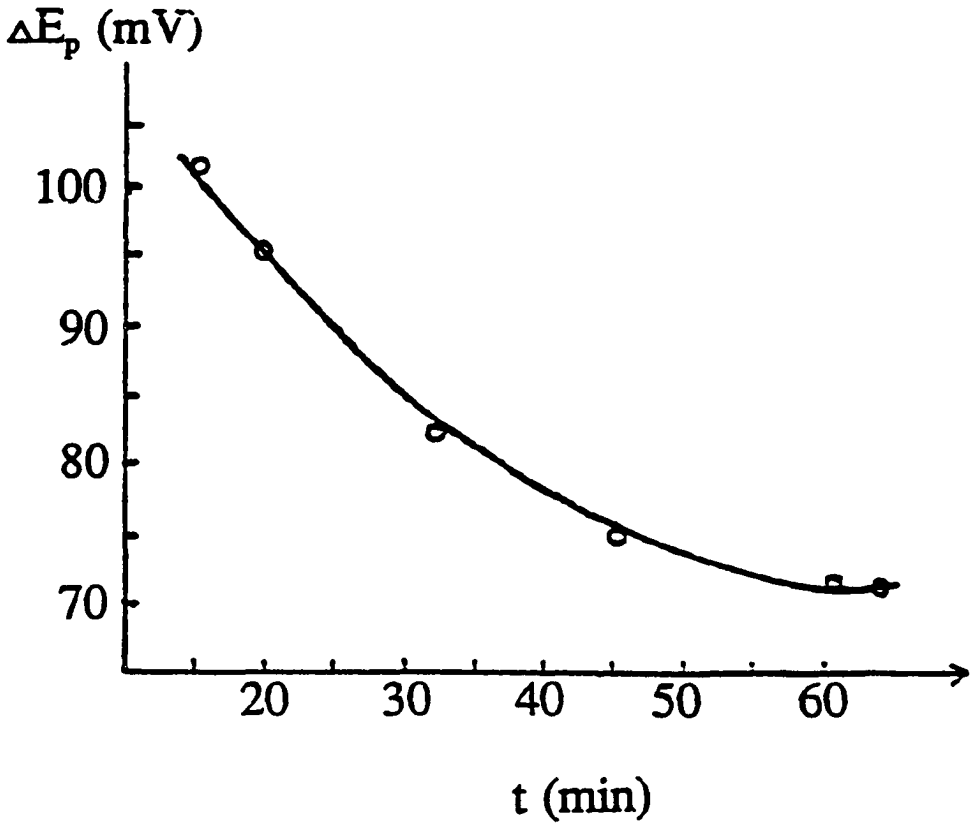

Figure 2. Dependence of the peak separation ΔE_p in the cyclic voltammograms of cytochrome c on the dipping times used in the preparation of thiophene-modified gold electrodes.

cytochrome c on the dipping times in the modification procedure. It can be noted that the ΔE_p decreases with increase in the dipping time. For example, a ΔE_p of more than 100 mV was observed for a dipping time of just a few minutes. For dipping times of more than 1 h ΔE_p reached a stable value, 72 mV.

Figure 3 shows the S_{2p} peaks in the x-ray photoelectron spectroscopy spectra of thiophene adsorbed on surfaces of the gold electrodes for the different dipping times in modification procedure. It can be seen from Figure 3 that the peak intensity becomes greater with increasing dipping time, indicating that the amount of thiophene adsorbed on the electrode surface increases.

The behavior of carbazole and pyridine as promoters were different from that of thiophene. A saturated carbazole solution was used to modify the gold electrode because of the low solubility of carbazole in aqueous media. When a freshly polished gold electrode was dipped in the saturated solution of carbazole for less than 30 minutes, no response was observed in the cyclic voltammogram of cytochrome c, indicating that carbazole does not act as a promoter under these conditions. However, when the dipping time was increased to more than one hour, a pair of redox peaks was observed in the CV. In addition, the separation between the cathodic and anodic peak potentials (ΔE_p) decreased with an increase in the dipping time and reached a stable value for dipping times greater than 12 hours (Figure 4). The ΔE_p in the CV of cytochrome c at the carbazole-modified gold electrode prepared by dipping the electrode in carbazole solution for 12 hours was 74 mV at a scan rate of 50 mV/s. The heterogeneous electron transfer rate constant, k_s, was determined to be 4.54×10^{-3} cm/s. The midpoint between the

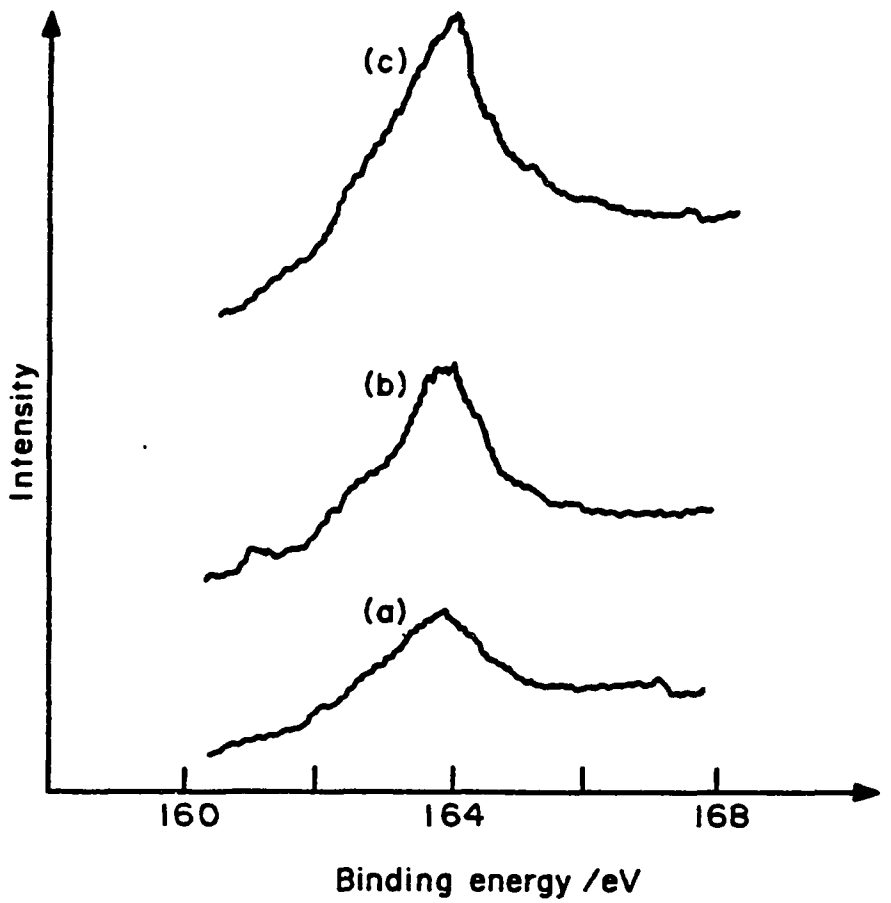

Figure 3. Dependence of the intensity of the S_{2p} peak in the XPS spectra of thiophene adsorbed on the surface of the gold electrode on the dipping time of modification procedure. The dipping times were as follows: (a) 15 minutes, (b) 30 minutes, (c) 45 minutes.

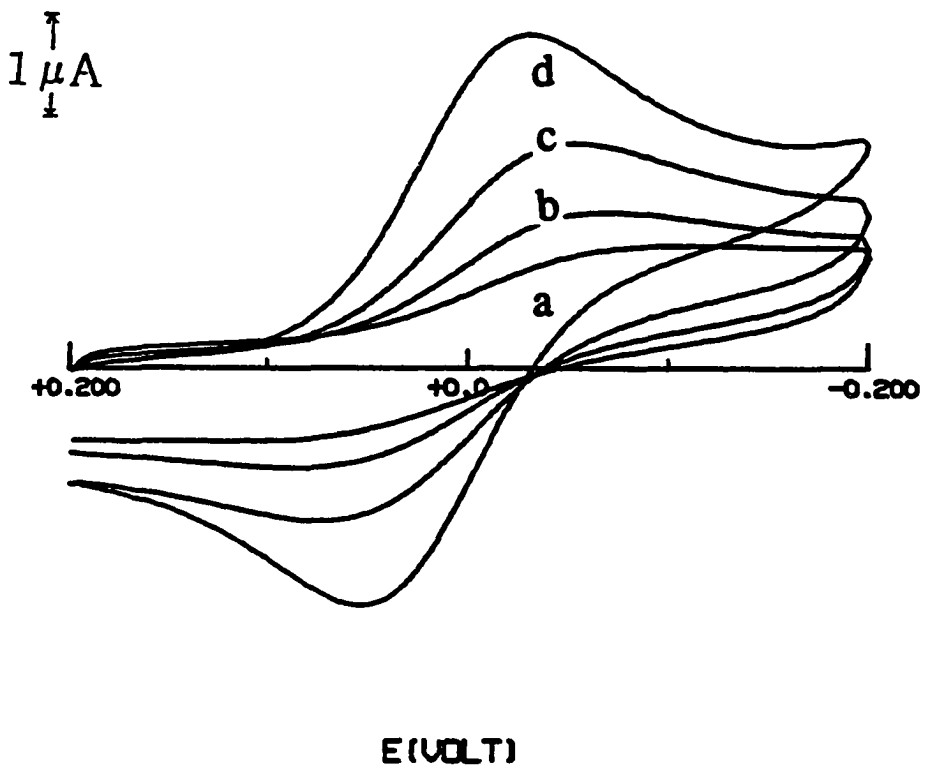

Figure 4. Cyclic voltammograms of 0.38 mM cytochrome c at a carbazole-modified gold electrode in phosphate buffer solution (pH 7.0) with 0.1 M $NaClO_4$. The dipping times were as follows: (a) 2 minutes, (b) 30 minutes, (c) 6 h, (d) 12 h. The scan rate was 50 mV/s and the initial potential was +0.2 V.

cathodic and anodic peak potentials was approximately +0.01 V, which is close to the formal potential of cytochrome c [10]. The cathodic and anodic peak currents were proportional to the square root of the scan rate in range 10-500 mV/s, indicating that the reaction is diffusion controlled. The performance of the carbazole-modified gold electrode was not very stable. The ΔE_p in the CV of cytochrome c at carbazole-modified gold electrode increased slightly with continuous cycling. For example, the ΔE_p for the first cycle was 74 mV, whereas it was 88 mV after 25 continuous cycles.

The behavior of pyridine as a promoter was similar to that of carbazole. For example, the dependence of ΔE_p on the dipping time for pyridine was also observed. However, the ΔE_p in the CV of cytochrome c at the pyridine-modified gold electrode prepared with a 12 hour dipping time was 134 mV which is much larger than that for carbazole. The corresponding k_s is 7.01×10^{-4} cm/s. The performance of the pyridine-modified gold electrode was also not stable. After 25 continuous cycles, the ΔE_p was greater than 160 mV (Figure 5). These results indicate that although pyridine can function as a promoter, it is not as effective as carbazole.

Discussion

From the results described above, it can be concluded that compounds with only one functional group, such as thiophene, carbazole and pyridine, can function as electron transfer promoters and accelerate the electrochemical reaction of cytochrome c at gold electrodes.

The results of this investigation provide information regarding the possible mechanism for the promoter effect. The mechanism for the quasi-reversible, direct

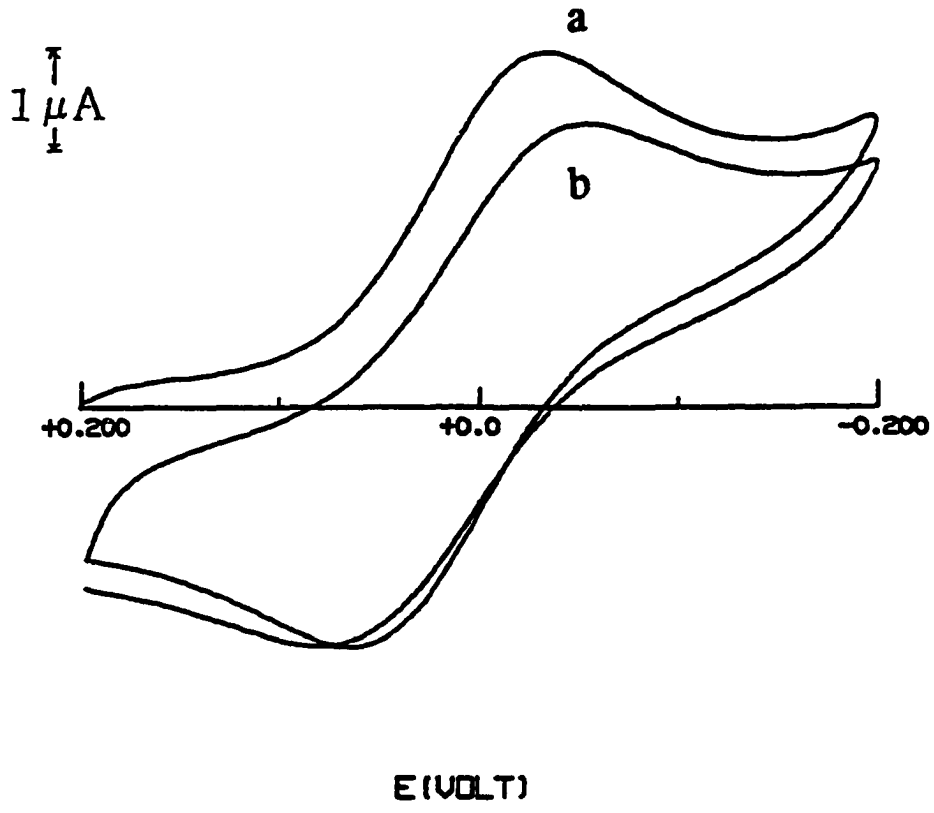

Figure 5. Cyclic voltammograms of 0.38 mM cytochrome c at a pyridine-modified gold electrode in phosphate buffer solution (pH 7.0) with 0.1 M $NaClO_4$. The dipping time was 12 h. (a) the first cycle, (b) the 25th cycle. The scan rate was 50 mV/s and the initial potential was +0.2 V.

electrochemistry of cytochrome c at the promoter-modified metal electrodes is not well understood. Several possible mechanisms have been proposed. Hill and his coworkers suggested [6,7] that the organic promoters should be bifunctional. One functional group of the promoter binds to the electrode surface and the second interacts with lysine $-NH_3^+$ groups of cytochrome c through hydrogen bonding or salt bridging. An electron transfer pathway is thus formed between cytochrome c and the electrode surface and the electrochemical reaction of cytochrome c is accelerated. In addition, after the study of the electron transfer reaction of cytochrome c at a 4,4'-bipyridine-modified gold electrode, Hill concluded [11] that rapid adsorption and desorption of cytochrome c on the electrode surface is necessary for fast electron transfer. Niki and coworkers [12,13] demonstrated that the promoter can prevent adsorption of denatured cytochrome c on the surface of the metal electrode which results in the irreversible electrochemical reaction of cytochrome c. Surface enhanced resonance Raman spectroscopy of cytochrome c [14] also indicated that direct adsorption of cytochrome c on the surface of the metal electrode leads to a structural change of cytochrome c and rupturing or weakening of the methionine bond, while cytochrome c adsorbed on the modified electrode surface retains its native structure. It was suggested the electrostatic interaction between cytochrome c and the surface of the promoter-modified metal electrode may play an important role in the electrochemical response [15-18]. The relationship between cytochrome c purity and its electrochemistry was first reported by Bowden et al. [19]. Recently, Taniguchi et al. [20] and Daido et al. [21] found that irreversible adsorption of the deaminated and oligomeric components in cytochrome c samples from commercial sources on the electrode surface is responsible for

the irreversible electrochemical reaction of cytochrome c.

The results of this investigation demonstrated that thiophene, carbazole and pyridine are effective promoters. They all have only one functional group, >S, >NH, >N respectively, through which the promoter molecules are adsorbed on the electrode surface. Thus, it appears that at least one class of promoters does not require a structure with two functional groups and it is not necessary for accelerating the electrochemical reaction of cytochrome c that the promoter molecules interact with cytochrome c molecules through hydrogen bonding or salt bridging to form an electron transfer pathway between electrode and cytochrome c [6,7]. Therefore, the crucial role of the promoters may be to prevent denaturation and/or irreversible adsorption of cytochrome c or the deaminated and oligomeric components, which results in irreversible electrochemistry. The electrostatic interaction between cytochrome c and electrode surface may also be important. The surface modification of the metal electrodes with promoter may change the charge on the electrode surface. The shift in the open circuit potential after surface modification of the electrode with thiophene is evidence for a more negative charge on the electrode surface after modification which favors the reversible electron transfer between cytochrome c and the electrode [15].

When the film transfer method is used to study the direct electrochemistry of cytochrome c at a promoter-modified metal electrode a dipping time of a few minutes is is commonly used for surface modification of the electrode [4-6]. The dependence of the ΔE_p on the dipping time was first reported by our group [8]. Dipping times as long as 10 h are required to observe the effective promoter effects of 2,2'-bipyridine and pyrazine

which are weakly adsorbed on the gold surface. In this study, it was shown that the dependence of the ΔE_p on the dipping time was also observed for thiophene, carbazole and pyridine. The intensity of the S_{2p} peak in the XPS spectra of thiophene adsorbed on the gold surface increases with increasing dipping time, indicating that the amount of the promoter molecules adsorbed also increases. In turn, ΔE_p is dependent on the amount of promoter molecules adsorbed on the electrode surface. One possible reason is that the more promoter molecules adsorbed on the electrode surface, the less the bare electrode surface and the better the prevention of the denaturation and/or irreversible adsorption of cytochrome c. Thus, it is better to use a prolonged dipping time in the modification procedure for evaluation of the promoter ability of compounds.

In previous studies [3-6], two methods were used to evaluate the promoter ability of a compound. In one method, the electrochemical measurement is carried out in the cytochrome c solution containing the promoter to be tested. The second method, the film transfer method, was used in this work. It should be noted that when the film transfer method is used to evaluate the promoter ability of compounds, there are two types of promoters. For the first type of promoters, such as thiophene, whose molecules can be strongly adsorbed on the gold surface, a dipping time of about 1 h is sufficient to observe the optimum promoter effect and the performance of the thiophene-modified gold electrode is very stable. For the second type, such as 2,2'-bipyridine, pyrazine [8], carbazole and pyridine, which are weakly adsorbed on the electrode surface, dipping times as long as 10 h are required to observe their optimum promotion effect. In addition, the performance of the modified electrodes is not stable because the weakly adsorbed promoter molecules

are gradually replaced by cytochrome c molecules from solution. Once cytochrome c molecules are directly adsorbed on the electrode surface, they undergo denaturation and block electron transfer with the cytochrome c molecules in solution and the CV response declines rapidly.

Conclusions

Molecules with one functional group, such as pyridine, carbazole and thiophene can also accelerate the electrochemical reaction of cytochrome c. However, their effectiveness as promoter is somewhat different because of their different adsorption strengths on the surface of the gold electrode. The behavior of the different promoters with one functional group indicates that an important requirement for an effective promoter is that it interacts with the electrode surface. The crucial role of these three promoters may be to prevent denaturation and/or irreversible adsorption of cytochrome c or the deaminated and oligomeric components, which results in irreversible electrochemistry.

References

1. C. Hinnen, R. Parsons and K. Niki, *J. Electroanal. Chem.*, **1983,** 147, 329.

2. R. A. Morton and K. Breskvar, *Can. J. Biochem.*, **1977,** 55, 146.

3. M. J. Eddowes and H. A. O. Hill, *J. Chem. Soc. Chem. Comm.*, **1977,** 771.

4. I. Taniguchi, K. Toyosawa, H. Yamaguchi and K. Yasukouch, *J. Electroanal. Chem.*, **1982,** 140, 187.

5. I. Taniguchi, M. Iseki, K. Toyosawa, H. Yamaguchi and K. Yasukouch, *J. Electroanal. Chem.*, **1984,** 164, 385.

6. P. M. Allen, H. A. O. Hill and N. J. Walton, *J. Electroanal. Chem.*, **1984,** 178, 69.

7. F. A. Ammstrong, H. A. O. Hill and N. J. Walton, *Acc. Chem. Res.*, **1988**, 21, 407.

8. C. Zhou, S. Ye, T. M. Cotton, X. Yu, T. Lu and S. Dong, *J. Electroanal. Chem.*, **1991**, 319, 71.

9. R. S. Nicholson, *Anal. Chem.*, **1965**, 37, 151.

10. R. W. Henderson and W. R. Rawlinson, *Biochem.*, **1956**, 62, 21.

11. W. J. Alberry, M. J. Eddowes, H. A. O. Hill and A. R. Hillman, *J. Am. Chem. Soc.*, **1981**, 103, 3904.

12. T. Sagara, K. Niwa, A. Sone, C. Hinnen and K. Niki, *Langmuir*, **1990**, 6, 254.

13. T. Sagara, H. Murakami, S. Igarahsi, H. Sata and K. Niki, *Langmuir*, **1991**, 7, 3190.

14. C. Zhou, T. M. Cotton, X. Qu, T. Lu and S. Dong, in *Redox mechanisms and interfacial propoerties of molecules of biological importance*, V. F. Schultz, I. Taniguchi, (eds), p 63-74, Electrochemical Society, Pennington, NJ, **1993.**

15. T. Lu, X. Yu, S. Dong, C. Zhou, S. Ye and T. M. Cotton, *J. Electroanal. Chem.*, **1994**, 369, 79.

16. C. Van Dijk, J. W. Van Leeuwen and C. Veeger, *Bioelectrochem. Bioenerg.*, **1982**, 9, 743

17. F. A. Armstrong, H. A. O. Hill, B. N. Oliver and D. Whitford, *J. Am. Chem. Soc.*, **1985**, 107, 1473.

18. A. Manjaoui, J. Haladjian and P. Bianco, *Electrochim. Acta*, **1990**, 35, 177.

19. E. F. Bowden, F. M. Hawkridge, J. F. Chlebowwski, E. F. Bancroff, C. Thorpe, and H. N. Blount, *J. Am. Chem. Soc.*, **1982**, 104, 7461.

20. I. Taniguchi, H. Kurihara and K. Yoshida, *Denki Kagaku*, **1992**, 60, 1043.

21. T. Daido, T. Akaiki, *J. Electroanal. Chem.*, **1993**, 344, 91.

CHAPTER 3. ELECTROCHEMICAL AND SURFACE-ENHANCED RESONANCE RAMAN BEHAVIOR OF CYTOCHROME C AT IODIDE-MODIFIED ELECTRODES

Introduction

Cytochrome c, an electron carrier between the cytochrome c reductase and cytochrome c oxidase, is widely distributed in living organisms. Its physiological redox partners are bound to the inner membrane of mitochondria, whereas cytochrome c itself resides in the cytosol between the inner and outer membranes [1] and is subject to very high electric fields (10^7-10^8 V/m) near the surface of the membrane [2]. A field of similar magnitude exists at an electrode/solution interface and, consequently the electrochemical behavior of cytochrome c is of considerable interest. However, early studies have shown that the electrochemical reactions of cytochrome c at metal electrodes, such as Hg, Pt, Au, Ag etc., are irreversible and sometimes not detectable [3] despite the fact that cytochrome c is readily oxidized or reduced by chemical reagents, (e.g. $NaNO_2$, Na_2SO_3 etc.). For this reason mediators were often employed in electrochemical studies of cytochrome c. In 1977 quasi-reversible, direct electron transfer was observed between cytochrome c and tin oxide electrodes [4] and at 4,4'-bipyridine-modified electrodes [5]. In the latter system, 4,4'-bipyridine was termed a "promoter" of the electron transfer process because it is not redox active within the potential range used to reduce or oxidize cytochrome c.

Following the initial observations of Hill and coworkers, an extensive research effort has been devoted to the study of the direct electrochemical reaction between cytochrome c and promoter-modified electrodes [5-31]. To date, most of the promoters that have been studied are organic compounds such as 4,4'-bipyridine [5], 1,2-bis-(4-

pyridyl)ethylene [5], bis-(4-pyridyl)disulfide [12] and purine [16]. Only a few inorganic promoters, such as S and As adatoms [26] and heteropolytungstates [30] have been reported. Gold electrodes were used most often in these studies, but quasi-reversible electron transfer behavior between cytochrome c and other promoter-modified metal electrodes, such as Pt [9] and Ag [14] etc. has also been reported. With respect to other biological compounds, Lane and Hubbard [32] reported a study of the electrochemical behavior of catecholamines which showed that modification of Pt electrodes with iodide ion produces a surface which is inert with respect to electrochemical and chemical interference over the potential range of interest. Adsorbed iodide prevented the formation of surface oxides on the Pt as well as adsorption of biological compounds. Detection of the dopamine was possible at the iodide modified electrode, whereas no current was discernible at bare Pt owing to the high background current as well as the irreversible nature of the electron transfer process.

The mechanism for the heterogeneous electron-transfer process between cytochrome c and promoter-modified electrodes is not well understood. It has been shown that cytochrome c is structurally altered (i.e. unfolded or flattened) when adsorbed directly on metal electrodes and under these conditions it undergoes irreversible electron transfer [33-36]. It may be that promoters somehow prevent structural changes in cytochrome at the electrode surface. The majority of promoters studied so far are bifunctional. One functional group binds to the electrode surface and the second is believed to interact with lysine-NH_3^+ groups of cytochrome c through hydrogen bonding or salt bridging [17,21,25]. When bound to the promoter-modified electrodes cytochrome c maintains its native

structure and undergoes quasi-reversible electron transfer [35]. Niki and coworkers [35] have shown that three types of interactions can occur between cytochrome c and organic modifiers. Also bifunctionality is not mandatory, as shown by our recent studies of monofunctional compounds which are effective promoters [36-38].

Hawkridge and coworkers have made extensive investigations of cytochrome c electrochemistry [39-42]. They found that after purification of cytochrome c and in the absence of lyophilization, a quasi-reversible electron transfer process can be observed at bare metal electrodes [40,41]. These authors suggested that the lyophilization process produces small amounts of oligomeric and polymerized cytochrome c and that these forms adsorb strongly onto the metal electrode surfaces. Electron transfer between bulk cytochrome c in solution and the electrode is irreversible under these conditions [40]. The heterogeneous electron transfer rates for small metalloproteins, such as cytochrome c_{553} [41] are greater than those for larger metalloproteins such as cytochrome c. Thus, it was concluded that the size of the biological molecule (i.e. the distance of closest approach between the heme edge and the electrode surface) controls the rates of the heterogeneous electron transfer of biological molecules at electrode surfaces [40-42].

Electrostatic interaction between cytochrome c and its redox partners are known to play an important role in homogeneous electron transfer reactions [43,44]. Several studies have emphasized similar electrostatic interactions between the metalloprotein and the electrode surface [45-47]. Surface-enhanced resonance Raman scattering (SERRS) studies of cytochrome c also provide support for protein adsorption on silver electrodes [48,49]. Moreover, Hildebrandt and Stockburger have shown that the spin state marker

bands in the SERRS spectra are sensitive to structural perturbations resulting from the interaction of the protein with the metal [49]. The high spin form of the protein was found to exhibit a more negative redox potential than the low spin form (-0.35 V versus 0.0 V). It was proposed that the orientation of the protein at the electrode surface changed with the adsorption potential as a result of electrostatic interactions between the charged amino acid groups on the surface of the protein and the electrode.

In the present study, it is shown that quasi-reversible, direct electron transfer occurs between cytochrome c and an iodide-modified gold electrode or between cytochrome c in an iodide-containing solution and a bare gold electrode. Direct evidence for adsorption of the protein on an iodide-modified Ag electrode was obtained from SERRS. These results support the role of electrostatic interactions in the electrochemistry of cytochrome c. A mechanism for the electrochemical reaction of cytochrome c at the iodide-modified electrode is proposed.

Experimental

Horse heart cytochrome c (type VI, Sigma Chemical Co.) was used without further purification. All other chemicals were reagent grade.

A BAS 100 electrochemical analyzer and conventional three-electrode electrochemical cell were used for the electrochemical measurements. The working electrode was constructed from a gold rod which was sealed into glass tubing with Torr seal (Varian). The exposed area was approximately 5.0 mm^2. A Pt wire was used as the auxiliary electrode. A saturated calomel electrode (SCE) served as the reference electrode and all the potentials are reported with respect to the SCE.

The modified electrodes were prepared by the following procedure. The working electrode was sequentially polished with 5, 0.3, 0.05 μm alumina/water slurries until a shiny, mirrorlike finish was obtained. The electrode was then sonicated in deionized water and washed thoroughly with deionized water. Surface modification of the gold electrode was carried out by dipping the freshly polished gold electrode into 0.1 M KI solution for two minutes, followed by rinsing twice with deionized water.

The electrochemical studies of cytochrome c were carried out at a freshly polished gold electrode in the 0.38 mM cytochrome c solution with KI or at the iodide-modified gold electrode in 0.38 mM cytochrome c solution without KI. The electrolyte solution contained 0.025 M phosphate buffer at pH 7.0 and 0.1 M sodium perchlorate. Oxygen was purged from solution by bubbling with nitrogen for 10 minutes prior to the electrochemical measurements.

Surface-enhanced resonance Raman scattering (SERRS) spectra were obtained from cytochrome c adsorbed on a roughened Ag electrode. The electrode was constructed from Ag wire and roughened by a double-potential-step oxidation-reduction cycle (ORC) as described previously [13]. Following the ORC, the electrode was dipped into a cytochrome c solution for 15 minutes. The electrode was removed, excess solution was shaken from its surface and it was placed in 0.025 M phosphate buffer solution (pH 7.0). In the case of the iodide modification, the roughened Ag electrode was dipped into 0.1 M KI for 2 minutes, removed from the solution and rinsed with deionized water. Cytochrome c was then adsorbed as described above for the bare Ag electrode. The SERRS spectra were recorded at room temperature. The 413.1 nm line of a Kr^+ laser was

used as the excitation wavelength and the power was 1 mW at the sample. The scattered light was collected in a backscattering geometry and focussed on the slit of a monochromator/spectrograph (Spex Triplemate 1377) equipped with 1200 lines/mm grating. The detector was an intensified diode array (model 1420, OMA III, EG&G Inc.).

Results

Cytochrome c does not undergo reversible electron transfer at a freshly polished gold electrode (Figure 1(a)). In contrast, a well-defined peak was obtained at an iodide-modified electrode (Figure 1(b)). The current is entirely due to cytochrome c; iodide anions exhibit no electrochemical response in the potential range from -0.2 V to +0.2 V. The separation between the cathodic and anodic peak potentials is about 70 mV, which is larger than that for a fully reversible one-electron transfer reaction. The cathodic peak current is almost the same as the anodic peak current. The midpoint between the cathodic and anodic peak potentials is approximately 0.01 V, which is close to the formal potential of cytochrome c [50]. The cathodic and anodic peak currents are proportional to the square root of the scan rate in the range 10-500 mV/s (Figure 2), showing that the reaction is diffusion-controlled.

From the slope of the plot of the cathodic peak current i_p versus the square root of the scan rate, $v^{1/2}$, the calculated diffusion coefficient of cytochrome c is 1.09×10^{-6} cm^2 s^{-1} which is in good agreement with that obtained at 4,4'-bipyridine- [5] or bis(4-pyridyl)disulfide- [12] modified gold electrodes. Using Nicholson's method [51], the heterogeneous electron transfer rate constant, k_s, was determined to be ca. 6.2×10^{-3} cm s^{-1} from the scan rate dependence of the peak separation. This value is larger than that

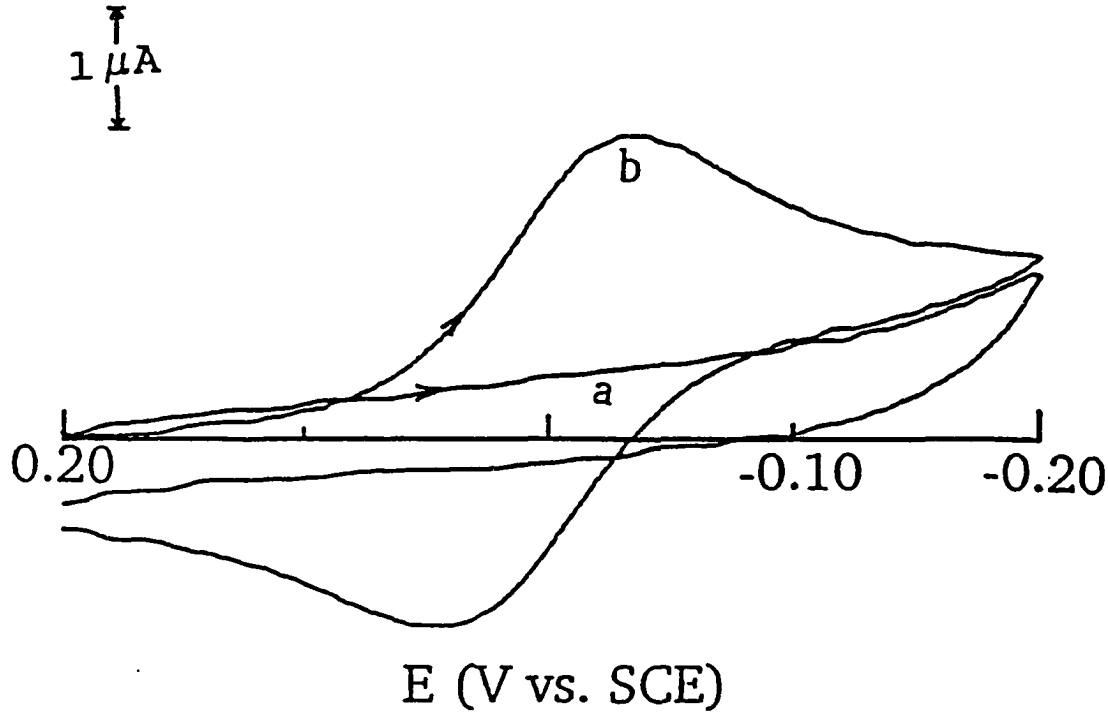

Figure 1. Cyclic voltammograms of 0.38 mM cytochrome c at (a) a freshly polished gold electrode, (b) an iodide anion-modified gold electrode in phosphate buffer solution (pH 6.97) with 0.1 M $NaClO_4$. Scan rate, 50 mV/s; initial potential, +0.20 V.

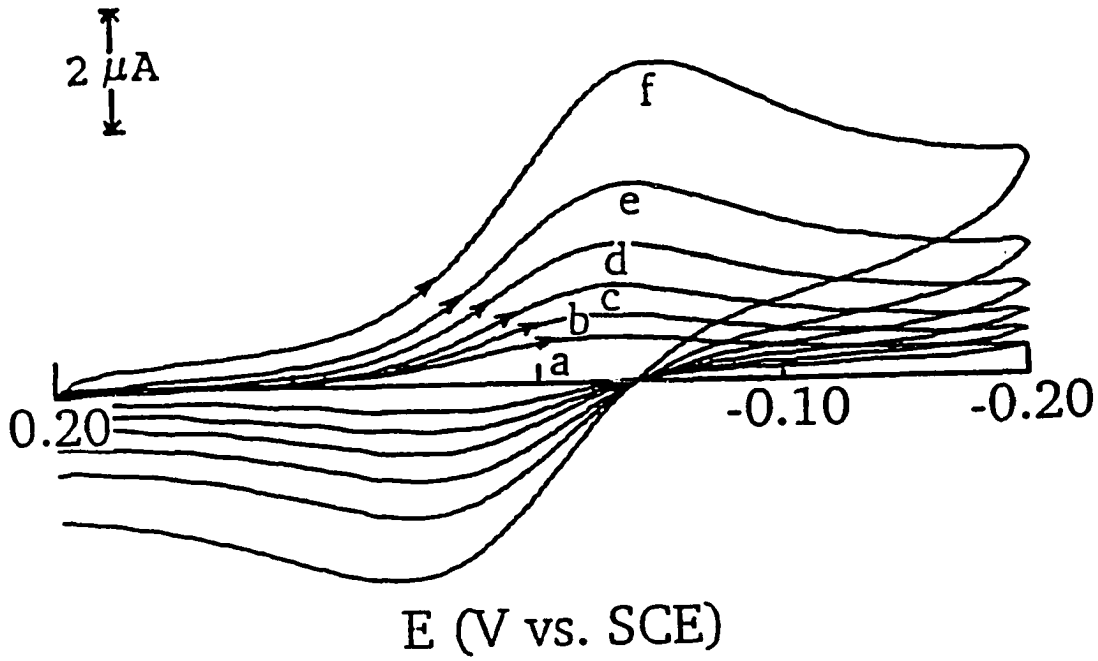

Figure 2. Cyclic voltammograms of 0.38 mM cytochrome c at an iodide-modified gold electrode in phosphate buffer solution (pH 6.97) with 0.1 M $NaClO_4$. Scan rates: (a) 10, (b) 25, (c) 50, (d) 100, (e) 200, (f) 500 mV/s. The initial potential was +0.20 V.

obtained at a purine-modified gold electrode (1×10^{-3} cm s^{-1}) [16], but less than that obtained at 4,4'-bipyridine-modified gold electrodes ($(1.4\text{-}1.9) \times 10^{-2}$ cm s^{-1}) [7].

All of the above characteristics are indicative of a quasi-reversible direct electron transfer reaction between cytochrome c and the iodide-modified gold electrode. Moreover, the performance of the iodide-modified gold electrode was very stable. After few days, the cyclic voltammetric response was almost the same as that observed initially.

The electrochemical behavior of cytochrome c was also studied at a freshly polished gold electrode in a cytochrome c solution containing varying concentrations of KI. The cyclic voltammograms were measured immediately after placing the freshly polished bare gold electrode into the cytochrome c solution. It was found that, under these conditions, the initial response depended upon the KI concentration in the cytochrome c solution. Curves a, b and c in Figure 3 show the response at a freshly polished gold electrode for cytochrome solutions containing KI concentrations of 3.8×10^{-4} M, 7.2×10^{-4} M and 3.8×10^{-3} M, respectively. It can be seen from Figure 3 that the peak current increases and peak separation decreases with increasing KI concentration. Figure 4 shows the relationship between the peak separation and a series of KI concentrations. The peak separation decreases sharply to a limiting value with increasing KI concentration. It was also noted that at low KI concentrations the peak separation decreased with time. For example, In the case of a 0.1 mM KI solution, the peak separation stabilized after approximately 1 hour, whereas in 1 mM KI solution, only 15 min was required. At the highest KI concentrations (10 mM) the peak separation reached a stable value within the time required to record a cyclic voltammogram. The peak current also increased with

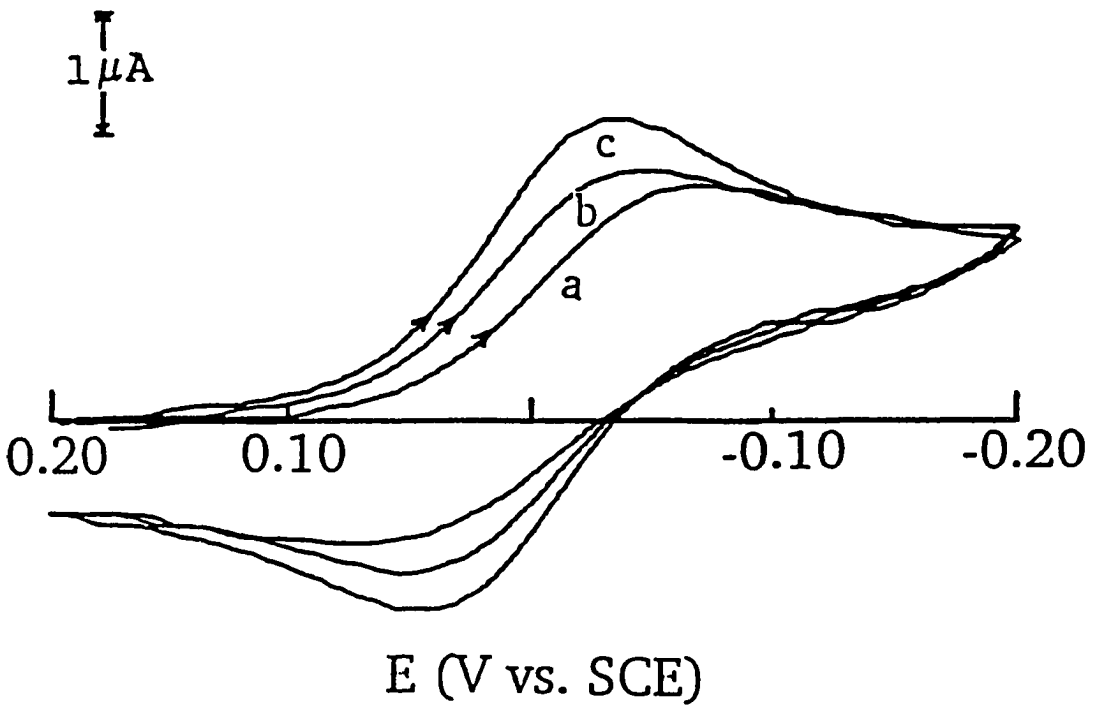

Figure 3. Cyclic voltammograms of 0.38 mM cytochrome c at a freshly polished gold electrode in phosphate buffer solution (pH 6.97) containing 0.1 M $NaClO_4$ and (a) 0.38, (b) 0.76, and (c) 3.8 mM KI. Scan rate, 50 mV/s; initial potential, +0.20 V.

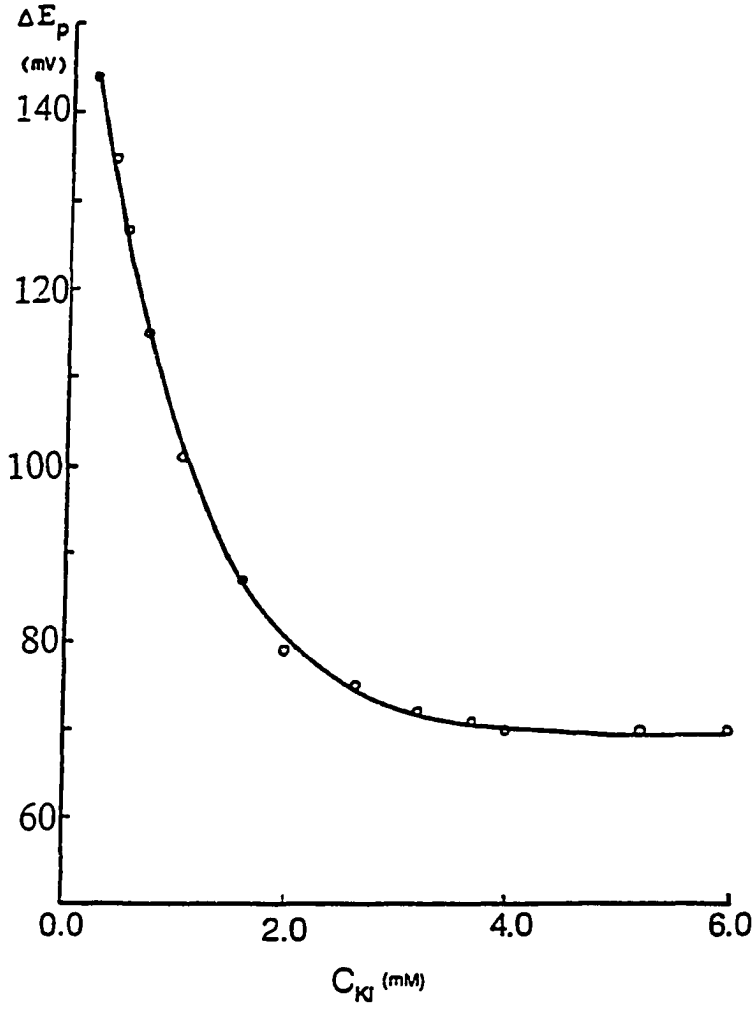

Figure 4. Plot of the peak separation ΔE_p as a function of the KI concentrations. Experimental conditions were the same as in Figure 3.

addition of KI, as shown in Figure 3. These results suggest that the iodide ion is adsorbed from solution onto the electrode surface and forms a stable modified electrode in the presence of cytochrome c. Any cytochrome c that is initially adsorbed is displaced by the iodide ion which is irreversibly adsorbed. The higher the iodide concentration, the more rapidly the cytochrome is displaced. In the absence of cytochrome c, the time required for adsorption of iodide from solution is also concentration dependent.

Surface-enhanced resonance Raman scattering was used to determine the conformations and redox properties of cytochrome c adsorbed on iodide-modified electrodes. In order to obtain surface enhancement on Au substrates it is necessary to excite at wavelengths near 600 nm. At this wavelength cytochrome c is not resonantly enhanced, however, and both resonance and surface enhancement are necessary to obtain sufficient scattering intensity from cytochrome c at the iodide modified electrode. For this reason, a silver electrode was used because both strong resonance and surface enhancement occur at 413 nm. The electrochemical response of cytochrome c at iodide-modified Ag electrodes was similar to that on iodide-modified Au electrodes, although the modification was not as stable as in the case of gold. A comparison of the SERRS spectra of cytochrome c adsorbed on the bare Ag electrode with those obtained on the iodide-modified Ag electrode demonstrates that the heme environment in the case of the latter more nearly resembles that of the native protein in solution. The position of the oxidation state sensitive band at 1372 cm^{-1} indicates that cytochrome adsorbed on the bare Ag electrode is in the oxidized state at the open circuit potential (data not shown). The spectra of cytochrome c adsorbed on a roughened Ag electrode at several potentials are

displayed in Figure 5. In the top spectrum (- 400 mV) the spin state marker band is split into two approximately equal bands at 1495 and 1474 cm^{-1} indicating that the heme group of the adsorbed protein is present as a mixture of five- and six-coordinate species. The adsorption interactions of the protein with the unmodified Ag surface lead to perturbation of the normal heme structure (six-coordinate, low spin), as noted in previous SERRS studies of cytochrome c [49,52]. These structural changes are also reflected in the redox potential of the protein. The band at 1365 cm^{-1}, the oxidation state marker band, is characteristic of the fully reduced heme and it shifts to 1377 cm^{-1} in the oxidized species. As can be seen from the changes in the oxidation state marker band, both the ferrous and ferric forms are present at -300 and -200 mV, indicating that the reduction potential is more negative than - 200 mV. The effect of potential on the frequency of the oxidation state marker band is plotted in Figure 7A. Although this curve is sigmoidal in appearance, it is quite broad and does not show a sharp change near the midpoint as expected from potentiometric measurements. This suggests that a mixture of species is present on the electrode surface in agreement with the SERRS results, as determined from the spin state marker band. An approximate value for the average reduction potential is - 0.25 V vs SCE as obtained from the midpoint of this curve. This is considerably more negative than the solution value, + 0.01 V vs SCE [50], for cytochrome c as measured by potentiometry. The negative shift indicates that the oxidized form of the cytochrome c is more stable in the adsorbed state and is characteristic of structural perturbations leading to a greater exposure of the heme group to the aqueous environment.

Figure 6 illustrates the changes observed in the SERRS spectrum of cytochrome

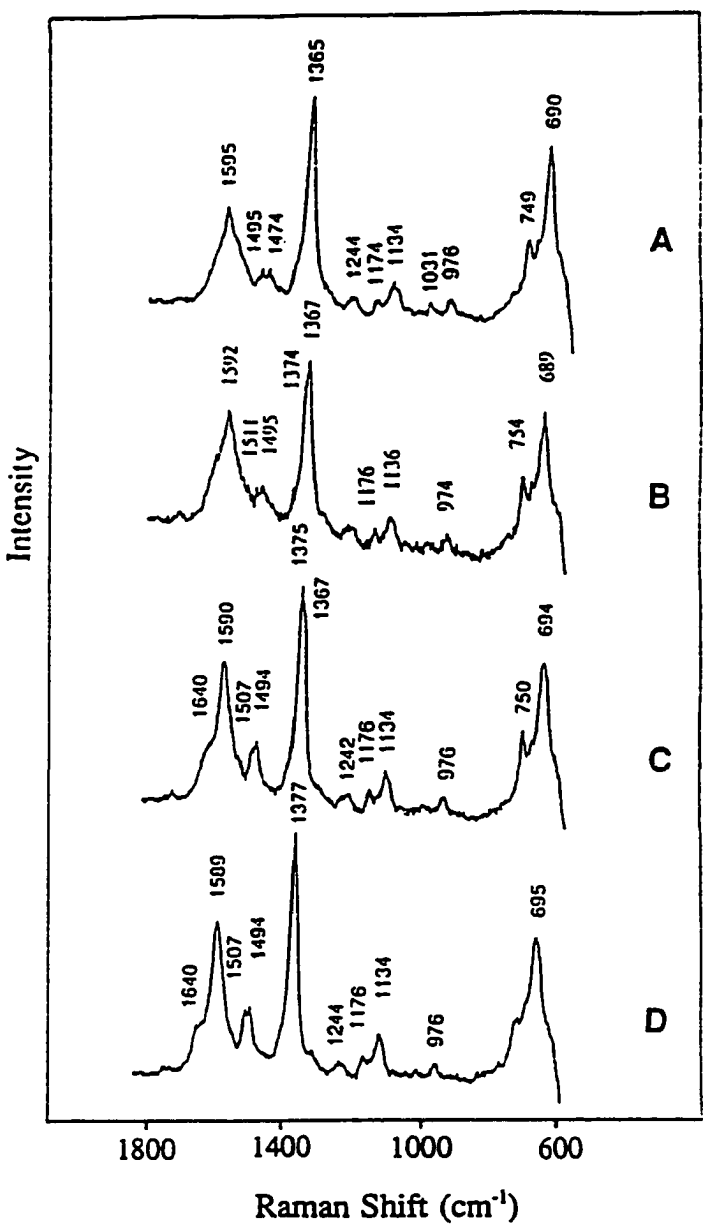

Figure 5. SERRS spectra of cytochrome c on a Ag electrode as a function of electrode potential: A) -400 mV; B) -300 mV; C) -200 mV and D) +80 mV. Experimental conditions were as follows: laser excitation wavelength = 413.1 nm; laser power = 1 mW; exposure time = 8 s/scan; number of scans accumulated = 20.

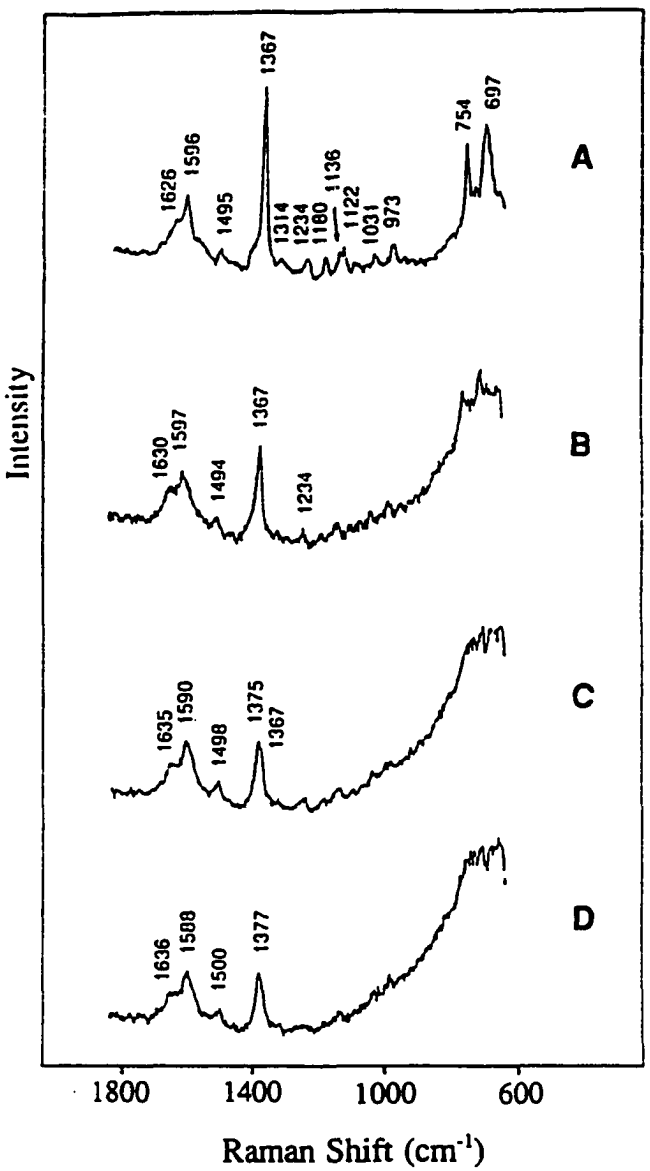

Figure 6. SERRS spectra of cytochrome c on an iodide-modified Ag electrode as a function of electrode potential: A) -400 mV; B) -200 mV; C) -50 mV and D) 0 mV. Experimental conditions were the same as in Figure 5.

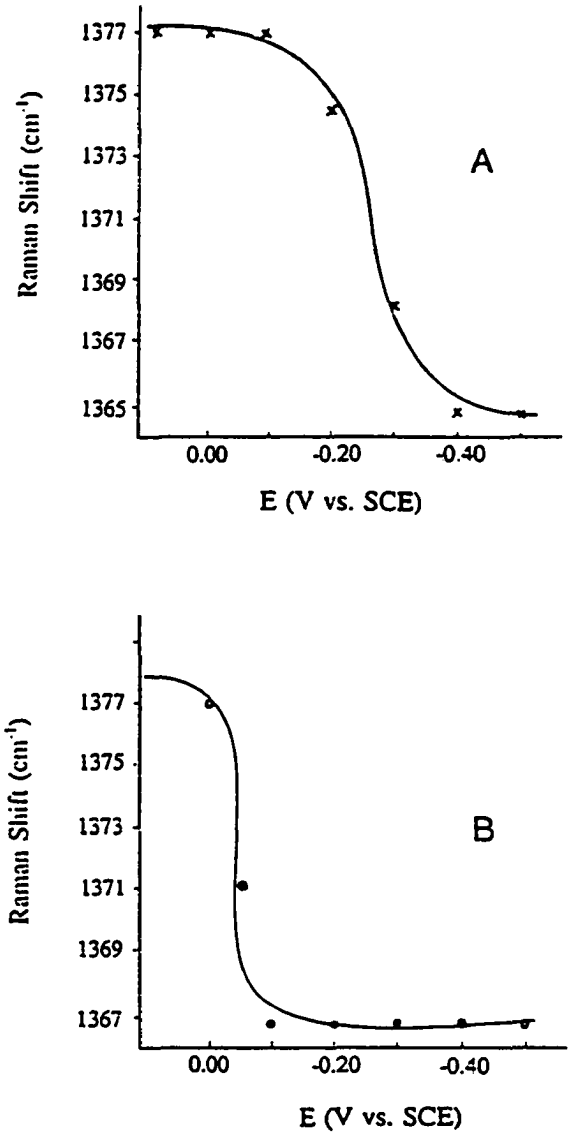

Figure 7. Variation in the position of the oxidation state marker band in the SERRS spectrum of cytochrome c as a function of the electrode potential. Figure A is for cytochrome c on a bare Ag electrode. Figure B is for cytochrome c on an iodide-modified Ag electrode.

c adsorbed on an iodide-modified Ag electrode. It should be emphasized that these spectra are from adsorbed cytochrome c only-no cytochrome c is present in the solution. At open circuit potential, the cytochrome c at iodide-modified Ag electrode is reduced (data not shown). Reduction of the cytochrome can be attributed to the shift in the rest potential of the iodide-modified Ag electrode from +0.08 V to -0.04 V, as determined experimentally. The first point to note in Figure 6 is that the spin state marker band is at 1495 cm^{-1} which is characteristic of the six-coordinate low spin form of the heme. The oxidation state marker band at 1367 cm^{-1} is shifted to 1377 cm^{-1} at 0 mV. At -200 mV, no evidence for the oxidized form can be seen, whereas a splitting is observed at -50 mV with the maximum at 1375 cm^{-1} and a shoulder at 1367 cm^{-1}. A full plot of the oxidation state marker band position as a function of potential is shown in Figure 7 B. In contrast to the curve shown for cytochrome c on a bare Ag electrode, this curve exhibits a sharp transition near the midpoint potential. A value of -0.05 V is obtained for the reduction potential. This value is very close to the electrochemical value obtained at the iodide-modified electrode by cyclic voltammetry, although it is somewhat negative relative to the solution value. Small negative shifts have been observed for cytochrome c adsorbed on In_2O_3 [36] and SnO_2 electrodes [37]. Similar shifts are also seen when cytochrome c interacts with biological components (mitochondrial membranes 50-60 mV) [53]. It can be concluded that the SERRS results indicate that the adsorbed cytochrome c is structurally similar to the native protein in solution. The spectrum decreases in intensity with repeated potential changes and this probably results from gradual desorption of the protein.

A second important point concerning Figure 6 is that the additional enhancement due to the resonance effect at 413.1 nm excitation is important for detecting the SERRS spectrum. The contribution of long range electromagnetic enhancement is apparent from the fact that only extremely weak spectra were observed at a roughened Au electrode with 413.1 nm excitation. Because the Au plasmon resonance is at longer wavelengths (> 600 nm), no surface enhancement is expected at this excitation wavelength. On the other hand, when 647.1 nm excitation was used with the Au electrode, the spectra were once again very weak because of lack of resonance enhancement at this wavelength. In summary, both resonance and surface enhancement are required to detect SERRS from cytochrome c adsorbed at modified electrodes.

Discussion

The above results show that adsorption of a simple anion, such as iodide, at a gold electrode can accelerate the heterogeneous electron transfer process between cytochrome c and a gold electrode. The electrostatic interaction between cytochrome c and the electrode surface appears to play a role in its electrochemical response. The sign and magnitude of the excess charge density at a metal electrode surface depends on the electrode potential with respect to the potential of zero charge (pzc) and the differential capacitance. The pzc for a gold electrode in the absence of specific ion adsorption is near +0.06 V [54] which is slightly more negative than that of the rest potential of the gold electrode in cytochrome c solution (+0.09 V). Therefore, a slight excess positive charge exists at the gold electrode surface at the rest potential. It has been reported [55,56] that iodide anions adsorb strongly and irreversibly on the gold surface. The forces responsible

for the strong interaction involve not only the simple coulombic attraction, but also covalent bonding between the iodide anion and the electrode surface, as shown by the SERS investigation of Gao and Weaver [56]. In our experiments, the iodide-modified gold surface is negatively charged at the rest potential (-0.05 V, as verified by direct measurement).

The possibility that iodide ions are adsorbed to specific sites on the cytochrome surface and that this is responsible for the enhanced electron transfer kinetics should also be considered. It is known that cytochromes bind a number of different anions and the ion binding properties vary widely with species [57]. Ion binding has been shown to have an effect on the redox potential of cytochromes from different species and the shift in potential has been analyzed in term of the binding constants [58]. Assuming that iodide also binds to the protein, it is conceivable that this could influence the protein interaction with the electrode surface and, hence, the electron transfer kinetics. However, experimental evidence suggests that the direct interaction of the iodide ion with the electrode surface may play a more important role. *Ex situ* modification of the electrode results in a stable surface which, when transferred to an iodide-free solution of cytochrome c, exhibits quasi-reversible electron transfer kinetics. Under these conditions, it is not likely that the iodide ion desorbs from the electrode and associates with the cytochrome in solution to an appreciable extent based upon the previous results of Gao and Weaver [56]. These authors have shown that iodide ion remained bound to Au throughout the potential range from +100 to -900 mV.

A consideration of the structure of cytochrome c suggests the possible interactions

that may occur between the amino acid groups on its surface and a charged electrode. Cytochrome c is a highly ionic protein with a net charge of +9 in the oxidized state at pH 7.5. Most of the positively charged residues are on the left front of the protein surface. On the other hand, nearly all the negatively charged residues are located in the small area on the back surface of cytochrome c. This results in a large dipole moment (312 and 300 debye for the oxidized and reduced forms, respectively). The dipole axis through the positive and negative centers crosses the cytochrome c surface at phenylalanine-82 (front surface) and asparagine-103 (back surface), respectively. The angle between the heme plane and the dipole axis of cytochrome c is 33 degrees. Phenylalanine-82 is thus located near the solvent-accessible heme edge [59,60]. The heme group sits in a crevice surrounded by the polypeptide chain of 104 amino acids. The plane of the heme is approximately perpendicular to the molecular surface. The solvent-exposed surface corresponds to a very small proportion (0.6%) of the total molecular surface and the heme edge in the crevice is located approximately 0.3 nm below the molecular surface [61]. This region of the protein surface is surrounded by positively charged lysines and constitutes an electron-transfer domain for interaction with cytochrome c oxidase or reductase [62]. Both of these mitochondrial reaction partners of cytochrome c are negatively charged, so that the molecules are electrostatically oriented as they approach one other. Every collision is productive and the electron transfer rates between cytochrome c and its physiological reactants are close to diffusion-controlled even though the surface area of the heme edge is only 0.6% of the total surface of cytochrome c [63].

Based upon the above properties of cytochrome c, a mechanism for the

electrochemical reaction at gold electrode can be proposed. In the case of a freshly polished gold electrode, the surface charge is positive. The cytochrome molecules will tend to orient with the negatively charged back surface proximal to the electrode surface and the positively charged surface near the heme crevice distal to the electrode surface. In the extreme case, the cytochrome interacts at the negatively charged patch of amino acids and the heme is quite distant from the electrode surface, as depicted in Figure 8A. Under these conditions efficient electron transfer to the heme is prevented. When the gold electrode is modified with iodide, the surface becomes negatively charged. Cytochrome c is adsorbed with the positively charged region of the protein surface closest to the electrode (Figure 8B). The heme group is proximal to the electrode surface and quasi-reversible electron transfer is observed.

The SERRS results support this interpretation. In the presence of adsorbed iodide ion, the heme exists in the normal low spin, six-coordinate state, whereas at the bare Ag electrode both high spin, five-coordinate and low spin, six-coordinate forms are present. Adsorbed cytochrome is in the oxidized state at bare Ag, as indicated by the position of the oxidation state marker band. It is reduced at the iodide-modified electrode because of the shift in the rest potential from +0.08 V to -0.04 V. This latter fact also indicates that the E^0 value of cytochrome at the iodide-modified electrode is closer to that of the protein in solution. It is known from previous potential dependent SERRS studies of cytochrome c at bare Ag electrodes that the redox potential is shifted by ca. -400 mV due to changes in the hydrophobicity of the heme environment and alterations of the protein structure near the heme [49]. Thus, it is apparent that in the presence of iodide these parameters are not

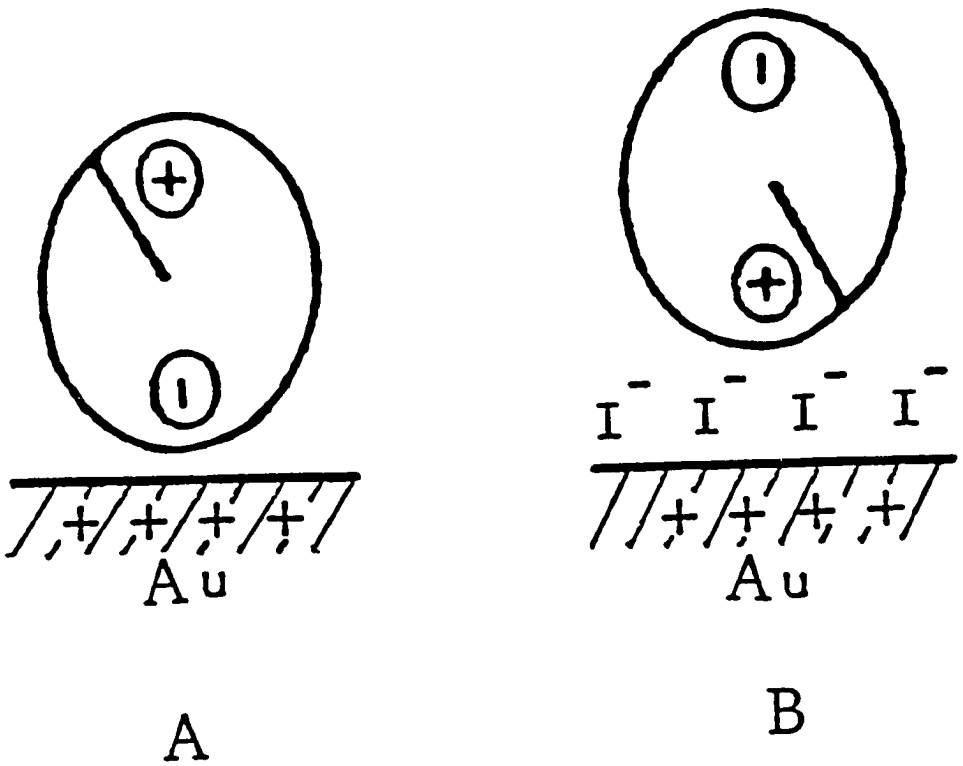

Figure 8. Schematic presentation of the orientation of cytochrome c adsorbed on a gold electrode surface in (A) the absence and (B) the presence of iodide anions. The large circles represent cytochrome c molecules; + and - signs in the circles represent the positive and negative centers of the dipole moment of cytochrome c; the line within the circle represents the heme plane.

affected to any significant extent.

These results are also in agreement with the model proposed by Hildebrandt and Stockburger [49] based upon their observation of two different cytochrome conformers. When cytochrome c was adsorbed at negative potentials (< -0.2 V vs. SCE) the heme was in the normal six-coordinate, low spin state, whereas when it was adsorbed at more positive potentials (> -0.2 V), the heme was in the five-coordinate, high spin state. They proposed that cytochrome c is attached to the metal via different groups of amino acids in the two conformers. In state II the electrostatic interactions are sufficiently strong to modify the coordination shell of the heme iron, whereas in state I, the interactions are much weaker and the heme is maintained in its normal coordination and spin state. These authors also noted an increase in the six-coordinate, low spin form in state II in the presence of Cl^-.

Albery et al. [8] have studied the kinetics of the electron transfer reaction of cytochrome c at a 4,4'-bipyridine-modified gold electrode. They concluded that rapid adsorption and desorption of cytochrome c is necessary for fast electron transfer. Consideration of the anion binding properties of cytochrome c in solution may provide some insight regarding its adsorption and desorption behavior at the iodide-modified electrode. Two factors must be considered: the number of ions bound as a function of redox state and the strength of ion binding. In one study the anion binding behavior was shown to be species dependent. Horse heart and bovine cytochrome were found to bind two chloride ions in the ferri form and three in the ferro form, whereas in the case of tuna cytochrome the situation was reversed [57]. However, the binding strength for chloride

was greater for the oxidized form than for the reduced. In another study, it was concluded that certain anions (i.e. those which are impermeable to the mitochondrial membrane) bind more strongly to the oxidized form of cytochrome c than to the reduced form, based upon electrophoretic mobilities of cytochrome c [56]. It may be that at an electrode surface specific binding sites on cytochrome c interact directly with the surface-adsorbed iodide and its adsorption/desorption behavior is affected by the changes in binding strength with redox state. Further experiments are needed to determine these parameters. However, the SERRS data obtained for cytochrome c on iodide-modified Ag electrodes do indicate that oxidized cytochrome c molecules are adsorbed more strongly than the reduced species. The SERRS spectrum of adsorbed cytochrome c was stable with time at positive potentials, whereas the signal decreased with cycling of the potential to negative potentials or when the potential was maintained at sufficiently negative values to reduce cytochrome c.

Conclusions

Quasi-reversible and direct electron transfer was observed between an iodide-modified gold electrode and cytochrome c, as well as between cytochrome c in an iodide-containing solution and a bare gold electrode. The results suggest that an electrostatic interaction between cytochrome c and the iodide-modified electrode surface plays an important role in the electrochemical response. Results obtained by surface enhanced resonance Raman scattering (SERRS) spectroscopy indicate that the heme group of the adsorbed cytochrome c is in the native low spin, six-coordinate configuration at the iodide-modified Ag electrode, whereas at the bare Ag electrode a mixture of both low spin, six-

coordinate and high spin, five-coordinate heme is present on the surface. A possible mechanism for the role of iodide as a promoter of cytochrome c electrochemistry is that the adsorbed iodide ions on the electrode surface change the surface charge of the electrode which leads to a favorable orientation of cytochrome c at the electrode surface for the electron transfer reactions.

References

1. Y. Huang and T. Kimura, *Biochemistry*, **1984**, 23, 2231.
2. S. Mclaughlin, in *Current Topic in Membrane transport*, F. Bronner and A. Kleiwieller, (Eds), Academic Press, New York, **1972**, Vol. 9, p. 71.
3. C. Hinnen and R. Parsons, K. Niki, *J. Electroanal. Chem.*, **1983**, 147, 329.
4. P. Yeh and T. Kuwana, *Chem. Lett.*, **1977**, 1145.
5. M. J. Eddowes and H. A. O. Hill, *J. Chem. Soc. Chem. Comm.*, **1977**, 771.
6. M. J. Eddowes and H. A. O. Hill, *J. Am. Chem. Soc.*, **1979**, 101, 4461.
7. M. J. Eddowes, H. A. O. Hill and K. Uosaki, *J. Electroanal. Chem.*, **1980**, 116, 527.
8. W. J. Albery, M. J. Eddowes, H. A. O. Hill and A.R. Hillman, *J. Am. Chem. Sco.*, **1981**, 103, 3904.
9. I. Taniguchi, T. Murakami, K. Toyosawa, H. Yamagucgi and K. Yasukouchi, *J. Electroanal. Chem.*, **1982**, 131, 397.
10. M. J. Eddowes and H. A. O. Hill, *Adv. Chem. Ser.*, **1982**, 201, 173.
11. J. Haladjian, R. Pilard, P. Bianco and P. A. Serre, *Bioelectrochem. Bioenerg.*, **1982**, 9, 91.

12. I. Tanguchi, K. Toyosawa, H. Yamaguchi and K. Yasukouchi, *J. Electroanal. Chem.*, **1982**, 140, 187.

13. R. Dhesi, T. M. Cotton and R. Timkovich, *J. Electroanal. Chem.*, **1983**, 154, 129.

14. T. M. Cotton, D. Kaddi and D. Iorga, *J. Am. Chem. Soc.*, **1983**, 154, 129.

15. V. Razumas, A. Samalius and J. Kulys, *J. Electroanal. Chem.*, **1984**, 164, 195.

16. I. Taniguchi, M. Iseki, K. Toyosawa, H. Yamaguchi and K. Yasukouchi, *J. Electroanal. Chem.*, **1984**, 164, 385.

17. M. P. Allen, H. A. O. Hill and N. J. Walton, *J. Electroanal. Chem.*, **1984**, 178, 68.

18. H. A. O. HIll, D. J. Page, N. J. Walton and D. J. Whitford, *J. Electroanal. Chem.*, **1985**, 187, 315.

19. B. A. Kuznetsov, G. P. Shumakovich and A. A. Mutuskin, *Bioelectrochem. Bioenerg.*, **1985**, 14, 347.

20. I. Taniguchi, T. Funatsu, K. Umekita, H. Yamaguchi and K. Yasukouchi, *J. Electroanal. Chem.*, **1985**, 199, 455.

21. D. Elliott, A. Hamnett, O. C. Lettington, H. A. O. Hill and N. J. Walton, *J. Electroanal. Chem.*, **1986**, 202, 303.

22. I. Taniguchi, N. Higo, K. Umekita and K. Yasukouchi, *J. Electroanal. Chem.*, **1986**, 202, 341.

23. Y. Gui and T. Kuwana, *J. Electroanal. Chem.*, **1987**, 226, 199.

24. K. Niwa, M. Furukawa and K. Niki, *J. Electroanal. Chem.*, **1988**, 245, 275.

25. F. A. Armstrong, H. A. O. Hill and N. L. Walton, *Acc. Chem. Res.*, **1988**, 21, 407.

26. M. Shibata and N. J. Furuya, *J. Electroanal. Chem.*, **1988**, 250, 201.

27. L. campanella, M. P. Sammartino, P. Stefanoni and G. Tranchida, *Bioelectrochem. Bioenerg.*, **1989**, 21, 55.

28. P. N. Bartlett and J. Farington, *J. Electroanal. Chem.*, **1989**, 261, 471.

29. S. Song, W. Zhang and S. Dong, *J. Inorg. Biochem.*, **1990**, 40, 189.

30. G. Chottard and D. Lexa, *J. Electroanal. Chem.*, **1990**, 278, 387.

31. C. Zhou, S. Ye, J. H. Kim, T. M. Cotton, X. Yu, T. Lu and S. Dong, *J. Electroanal. Chem.*, **1991**, 319, 71.

32. R. F. Lane and A. T. Hubbard, *Anal. Chem.*, **1976**, 48, 1287.

33. H. Berg, *Bioelectrochem. Bioenerg.*, **1977**, 4, 522.

34. P. A. Serre, J. Haladjian and P. Bianco, *Biopolymers*, **1982**, 21, 1781.

35. T. Sagara, H. Murakami, S. Igarahsi, H. Sata and K. Niki, *Langmuir*, **1991**, 7, 3190.

36. C. Zhou, X. Qu, T. Lu, S. Dong and T. M. Cotton, *Chin. Chem. Lett.*, **1992**, 3(2), 133.

37. C. Zhou, T. M. Cotton, X. Qu, T. Lu and S. Dong, in *Redox Mechanisms and Interfacial Properties of Molecules of Biological Importance*, V. F. Schultz, I. Taniguchi, (eds.), p. 63-74, Electrochemical Society, Pennington, NJ, **1993**.

38. X. Qu, T. Lu, S. Dong, C. Zhou and T. M. Cotton, *Bioelectrochem. and Bioenerg.*, **1994**, 34, 153.

39. S. C. Sun, D. E. Reed, J. K. cullison, L. H. Richard and F. M. Hawkridge, *Mikrochim. Acta*, **1988**, 111, 97.

40. K. B. Koller, F. M. Hawkridge, G. Fague and J. LeGall, *Biochem. Biophys. Res. Commun.*, **1987**, 145, 619.

41. B. C. King and F. M. Hawkridge, *J. Electroanal. Chem.*, **1987**, 237, 81.

42. E. F. Bowden, F. M. Hawkridge and H. N. Blount, *J. Electroanal. Chem.*, **1984**, 161, 355.

43. D. C. Rees, *Proc. Natl. Acad. Sci. USA*, **1985**, 82, 3082.

44. M. A. Cusanovich, J. T. Hazzard, T. M. Meyer and G. Tollin, *J. Macromol. Sci. Chem.*, **1989**, A26, 433.

45. C. Van Dijk, J. W. Van Leeuwen and C. Veeger, *Bioelectrochem. Bioenerg.*, **1982**, 9, 743.

46. F. A. Armstrong, H. A. O. Hill, B. N. Oliver and N. J. Walton, *J. Am. Chem. Soc.*, **1984**, 106 921.

47. A. Manjaoui, J. Haladjian and P. Bianco, *Electrochim. Acta*, **1990**, 35, 177.

48. For a review, see T. M. Cotton, J-H Kim and G. D. Chumanov, *J. Raman Spectrosc.*, **1991**, 22, 729.

49. P. Hidebrandt and M. Stockburger, *Biochemistry*, **1989**, 28, 6710.

50. G. M. Schmid and M. E. Curley-Fiorino, *Encyclopedia of Electrochemistry of the Elements*, Vol. 4, Marcel Dekker, New York, **1975**, p. 87.

51. R. S. Nicholson, *Anal. Chem.*, **1965**, 37, 1351.

52. T. M. Cotton, S. G. Schultz and R. P. Van Duyne, *J. Am. Chem. Soc.*, **1980**, 102, 7960.

53. P. L. Dutton, D. F. Wilson and C-P. Lee, *Biochemistry*, **1970**, 9, 5077.

54. J. Clavilier and C. N. Van Huoong, *J. Electroanal. Chem.*, **1977**, 80, 101.

55. F.C. Anson, *Acc. Chem. Res.*, **1975**, 8, 400.

56. P. Gao and M. J. Weaver, *J. Phys. Chem.*, **1986**, 90, 4057.

57. E. Margoliash, G. H. Barlow and V. Byers, *Nature*, **1970**, 228, 723.

58. D. Gopal, G. S. Wilson, R. A. Earl and M. A. Cusanovich, *J. Biol. Chem.*, **1988**, 24, 11652.

59. J. D. Rush, J. Lan and W. H. Koppenol, *J. Am. Chem. Soc.*, **1987**, 109, 2679.

60. W. H. Koppenol and E. Margoliash, *J. Biol. Chem.*, **1982**, 257, 4426.

61. F. R. Salemme, *Annu. Rev. Biochem.*, **1977**, 46, 299.

62. A. G. Mauk, R. A. Scott, H. B. Gray, *J. Am. Chem. Soc.*, **1980**, 102, 4360.

63. R. Margalit and A. Schejter, *Eur. J. Biochem.*, **1973**, 32, 500.

CHAPTER 4. RESONANCE RAMAN AND SURFACE-ENHANCED RESONANCE RAMAN SPECTROSCOPIC AND ELECTROCHEMICAL STUDY OF CYTOCHROME C MUTANTS

A paper to be submitted to *Biospectroscopy*

Chengli Zhou, Emanual Margoliash and Therese M. Cotton

Abstract

Rat wild-type cytochrome c (WT) and its mutants, Tyr67Phe, Thr78Val, Asn52Ile, Asp50Arg, and His26Val were studied by cyclic voltammetry (CV), resonance Raman (RR), and surface-enhanced resonance Raman scattering (SERRS) spectroscopy. Both SERRS and CV results show that most of the cytochrome c mutants behave similarly to the wild-type cytochrome c at electrodes in term of reversibility except for the His26Val mutant. However, the so-called "water mutants" Tyr67Phe, Thr78Val, and Asn52Ile have much lower redox potentials than that of the wild-type cytochrome c which indicates that these mutations increase the stability of the protein. The resonance Raman spectra of the water mutants also show considerable differences from the wild-type, especially in the low frequency region which indicates structural changes in the heme/protein interactions. The surface-enhanced resonance Raman results indicate that the heme group of the adsorbed cytochrome c and its mutants are in the six-coordinate low spin (6cLS) configuration at bis(4-pyridyl) disulfide-modified-silver electrodes, whereas mixtures of both 6cLS and five-coordinate high spin (5cHS) heme configurations are present at bare silver electrodes. The structural basis for these results are discussed.

Introduction

Protein electrochemistry is a subject of great interest and has been extensively developed in recent years [1-9] due to the fact that there are some analogies between the electrode reaction of proteins and their biological electron transfer reaction. Such studies can yield important information not only about thermodynamic, kinetic and structural properties of proteins, but also provide novel insights into the electron transfer mechanism of proteins *in vivo*. Mitochondrial cytochrome c is one of the most extensively studied proteins because it plays important role in energy transduction and it is relatively stable under *in vitro* conditions. Its structural and chemical reactivity are well-characterized, and it provides a paradigm for other globular proteins [10]. Many studies have been focused on developing and understanding the structure/function relationships in redox proteins [2,4]. Evidence as to the role of specific amino acids has been gleaned from correlation of extensive data obtained from high resolution crystallography, 2-D-Nosey nmr spectroscopy and amino acid analysis of eukaryotic cytochromes [11,12]. Extensive studies of cytochromes labeled with redox active species have established that the intramolecular electron transfer kinetics are affected by the amino acid pathway [13,14]. It is also well-known that the redox potential of the heme is influenced strongly by the nature of the axial ligand to iron in heme-containing proteins, as well as the hydrophobicity of the heme environment [15,16]. Recently, site-directed mutagenesis of invariant amino acids near the heme, but not directly ligated to it, have established that these substitutions can have considerable effect on the protein stability [10].

Resonance Raman spectroscopy is a useful technique for characterization of the

structural features of proteins. It can be used to identify the presence or absence of particular structural and ligation interactions of the amino acids [17]. Therefore, it is very powerful method for correlating structure and function. It is also an extremely sensitive technique for selectively probing the heme moiety of cytochromes. Vibrational modes have been assigned that are sensitive to the oxidation and spin state of the heme iron, as well as to distortions in the heme imposed by the surrounding protein [18]. Surface-enhanced resonance Raman spectroscopy provides addition information concerning the stability, redox potential, and configuration of the protein on an electrode surface [19].

In the present study, several cytochrome c mutants have been characterized by cyclic voltammetry, RR and SERRS in order to determine how the mutations affect the redox potential, electron transfer kinetics and the stability of cytochrome c.

Experimental

The procedures for preparing the mutant cytochromes have been described previously [10]. Bis(4-pyridyl) disulfide (4-PyS)>98% purity was purchased from Aldrich Chemical Co. and was used as received. All other chemicals were reagent grade without further purification. The electrochemical measurements were carried out in a conventional three-electrode electrochemical cell using a BAS 100 electrochemical analyzer (Bioanalytical Systems). The working electrode were constructed from a flattened gold or silver rod which was sealed into glass tube using Torr Seal (Varian Co.). A Pt wire was used as the auxiliary electrode. A saturated calomel electrode (SCE) served as a reference electrode and all the potentials are reported with respect to the SCE. The working electrodes were sequentially polished with 5.0, 0.3, 0.05 μm alumina slurries in

water. The electrodes were sonicated in distilled water following each step of the polishing procedure. Modification of the surface was achieved by dipping the electrode into a 1 mM solution of 4-PyS for 10 minutes, followed by rinsing with distilled water and immersion into the electrochemical cell containing 10^{-4} M cytochrome c solution, 10 mM potassium phosphate buffer (pH 7) and 0.5 M KCl for subsequent electrochemical measurements.

For the SERRS measurements, after polishing, the silver electrode was roughened in 0.1 Na_2SO_4 by stepping the potential from -0.55 V to +0.55 V, where 25mC/cm^2 charge was allowed to pass, and then stepping back to -0.55 V to reduce the Ag^+. The electrode was then modified by the procedure described above for the electrochemical measurements. The SERRS studies at modified silver electrodes were accomplished by adsorbing the cytochrome c from 0.1 mM solution for 15 minutes, rinsing the excess solution from the electrode with buffer and immersing the electrode into 10 mM potassium phosphate buffer (pH 7) containing 0.5 M KCl as the electrolyte.

The RR and SERRS spectra were obtained by excitation with the 413 nm line of a Kr^+ laser. A backscattering geometry was used for collecting the scattered light. The Raman instrumentation has been described previously [20]. This includes a Triplemate 1377 spectrometer and a Princeton Instrument CCD detector (model LN1152) cooled to -120°. Typically, the RR and SERRS data were acquired with ca. 1 mW of power and a total acquisition time of less than 2 minutes. Indene was used to calibrate the Raman frequencies.

Results and Discussion

The molecular structure of cytochrome c is illustrated in Figure 1 [21]. The mutants examined in this study are marked in Figure 1 including the following: Tyrosine 67 taken to phenylalanine (Tyr67Phe), asparagine 52 taken to isoleucine (Asn52Ile), threonine 78 taken to valine (Thr78Val), aspartate 50 taken to arginine (Asp50Arg), and histidine 26 taken to valine (His26Val). Figure 2 shows the CV response for cytochrome c Tyr67Phe mutant at a 4-PyS-modified gold electrode at different scan rates. A plot of the peak current for both the cathodic and anodic peaks shows a linear increase as a function of the square root of the scan rate. The same behavior was observed for wild-type cytochrome c and all of the other mutants, demonstrating that the electrochemical reaction at 4-PyS-modified gold electrode is diffusion-controlled. The electrochemical results are summarized in Table 1. From the peak separations of Table 1 it can be seen that most of the cytochrome c mutants have similar electrochemical kinetics to the wild-type cytochrome c, except His26Val mutant. This is not surprising, since aspartic acid (Asp) 50, tyrosine (Tyr) 67, threonine (Thr) 78 and asparagine (Asn) 52 are internal amino acids and their substitution by other amino acids does not significantly change the protein surface structure and, hence, its interaction with the electrode. These results agree well with the hypothesis [2,4] that the promoter modified electrode surface does not recognize cytochrome c molecule very rigorously. The surface lysine residues around the exposed heme edge of cytochrome c, such as Lys 27 and Lys 72, have been suggested to be involved in binding to an electrode surface [22]. Since an electrode covered with a monolayer of bis(4-pyridyl) disulfide is an ideally hydrophilic surface, substitution of the

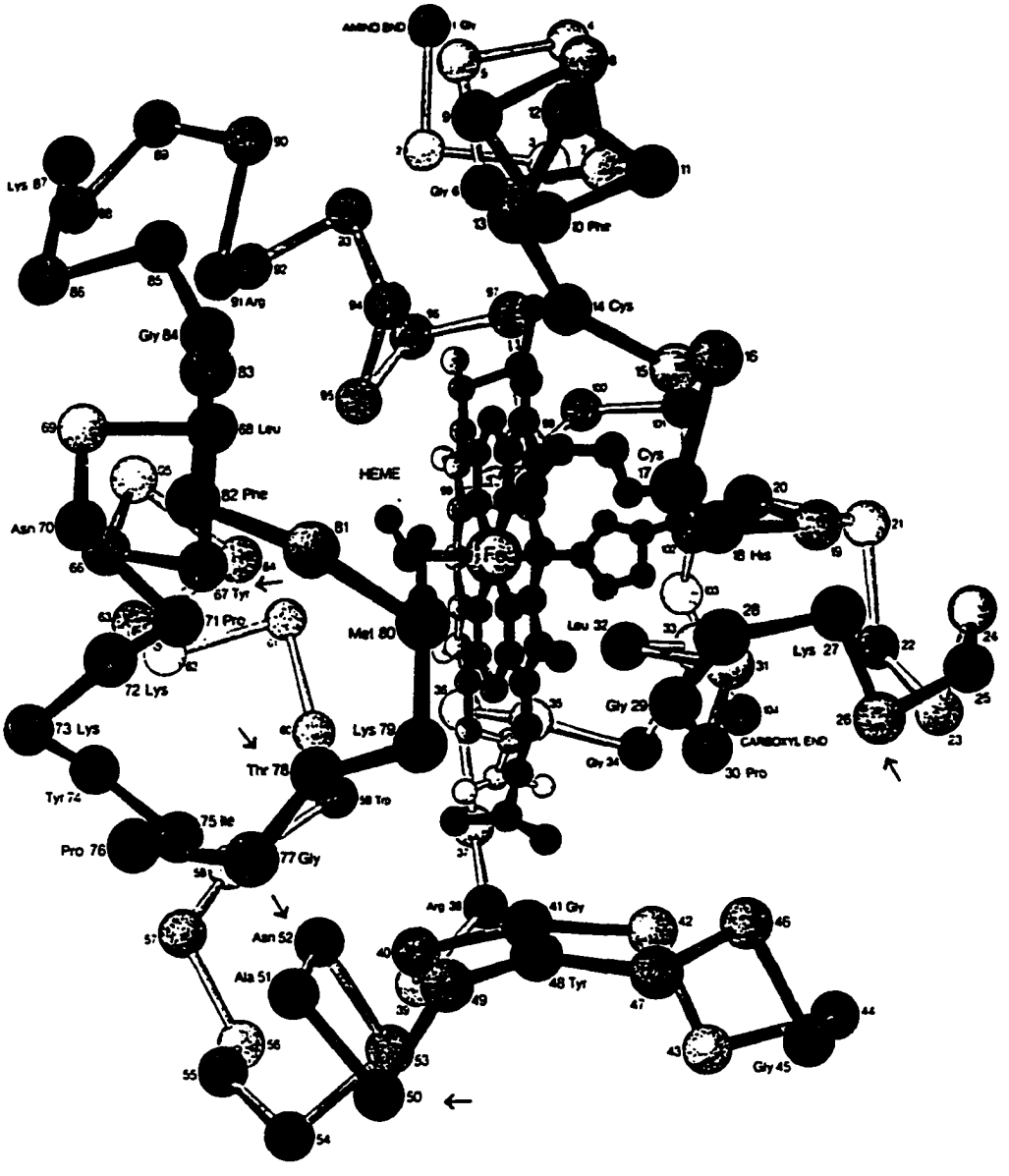

Figure 1. Molecular Structure of cytochrome c. This standard front view of horse heart cytochrome c in the oxidized state, illustrates the solvent exposed heme edge in the center of the molecule facing towards the reader. Only those amino acids attached directly to the heme (Met80, His18, Cys14, Cys17) are shown, all others are represented by circles depicting α-carbon atoms of the polypeptide backbone. All amide linkages are represented by a line between each α-carbon atom [14]. The mutation sites for this study are marked.

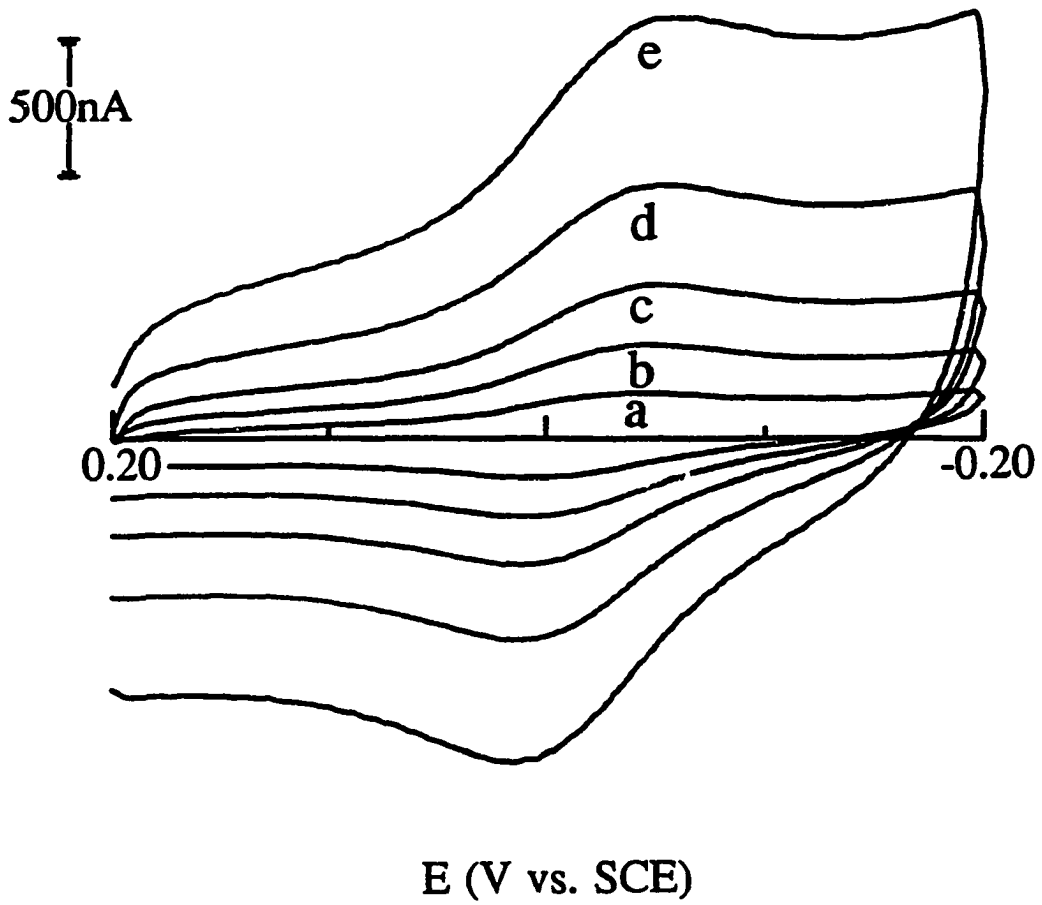

Figure 2. Cyclic voltammograms of 0.1 mM cytochrome c Tyr67Phe mutant at a 4-PyS-modified gold electrode in phosphate buffer solution (pH 7) with 0.5 M NaCl. Scan rate: (a) 10, (b) 25, (c) 50, (d) 100, (e) 200 mV/s; initial potential, +0.20 V.

Table 1: Redox potentials, peak separations and heterogeneous electron transfer rate constants of cytochrome c mutants based upon the CV data at a scan rate of 50 mV/s.

protein	redox potential (mV)	peak separation (mV)	electron transfer rate constant (10^{-3} cm/s)
WT	8.1	69.0	9.62
His26Val	-4.5	91.7	2.62
Asp50Arg	11.5	73.9	6.47
Tyr67Phe	-21.5	73.3	6.72
Asn52Ile	-30.1	74.5	5.42
Thr78Val	-----	-----	-----

polar histidine (His) 26 around the Lys 27 with a non-polar valine (Val) may weaken the interaction between the cytochrome c and the electrode surface and cause a decrease in the electron-transfer kinetics as indicated by the larger ΔE_p.

Figure 3 shows the SERRS spectra of Tyr67Phe mutant on both bare and modified silver electrodes at 0.0 mV. The spin state marker band at 1505 cm^{-1} in spectrum B indicates that the heme group of the adsorbed Tyr67Phe mutant is in the native low spin, six coordinate configuration at the 4-PyS-modified Ag electrode. In contrast, the presence of the split 1505 and 1493 cm^{-1} band at the bare Ag electrode indicates that a mixture of both low spin, six coordinate and high spin, five coordinate heme is present on the surface (spectrum A). The same behavior was also observed for the other cytochrome c mutants which emphasizes that the 4-PyS modifier plays an important role in stabilizing the native configuration of cytochrome c on the electrode surface.

Figure 4 shows the SERRS spectra of Tyr67Phe mutant on 4-PyS-modified silver electrode as a function of the electrode potential. The oxidation state marker band at 1361 cm^{-1} in spectra (a) and (b) show that at open circuit and -45 mV potentials, this mutant is fully reduced. The band is shifted to 1372 cm^{-1} when a potential of +30 mV is applied indicating that the cytochrome is fully oxidized. Figure 5 shows the potential dependence of the oxidation state marker in the SERRS spectra of the Tyr67Phe mutant. From the plot, the fully oxidized and reduced potentials and the estimated redox potential can be obtained. Table 2 shows summarized the results obtained for all the cytochrome c mutants. Comparing Table 2 with Table 1 it can be seen that the potential ranges of Table 2 are close to the peak separations of Table 1 for corresponding cytochrome c mutants;

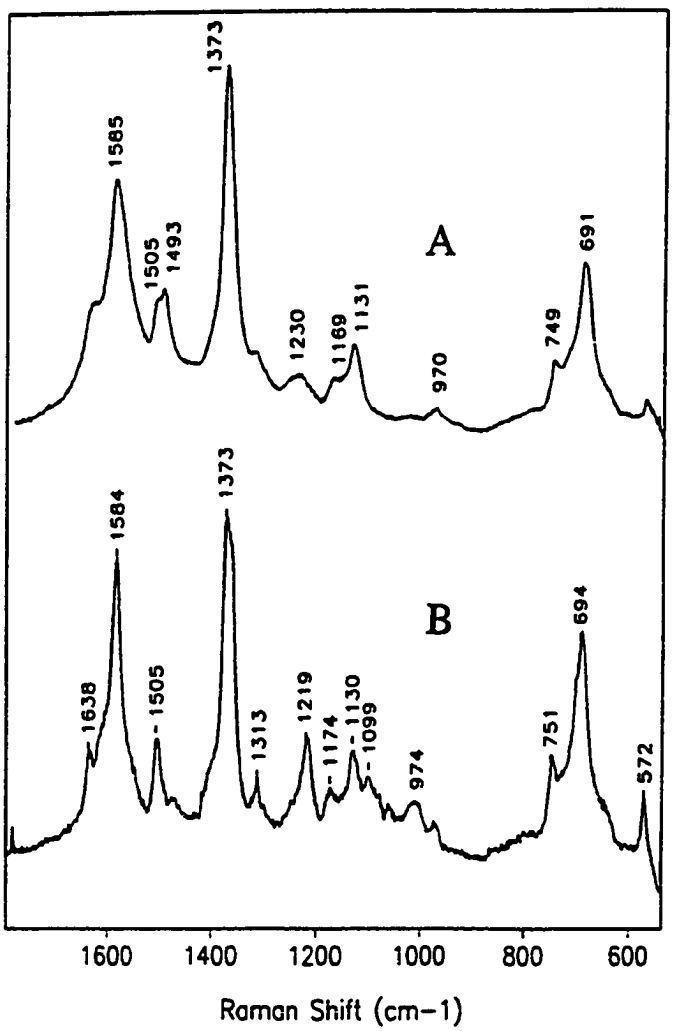

Figure 3. Surface-enhanced resonance Raman spectra of the cytochrome c Tyr67Phe mutant on (A) a bare silver electrode, (B) a 4-PyS-modified silver electrode at 0.0 mV vs. SCE. Experimental conditions were as follows: laser excitation wavelength = 413.1 nm; laser power = 1 mW; exposure time = 20 s/scan; number of scans accumulated = 20.

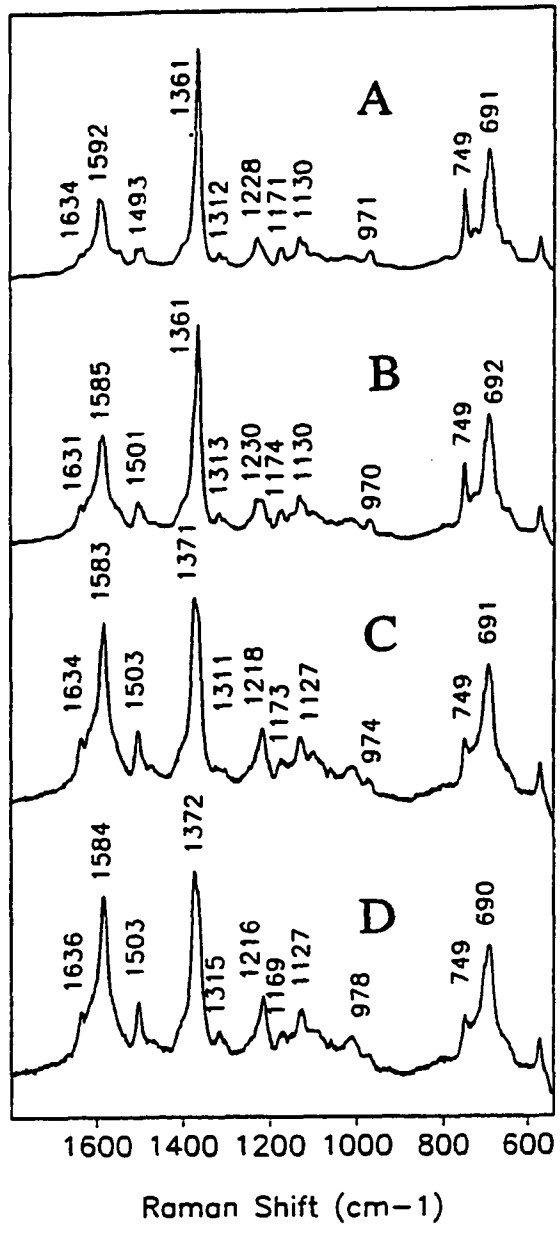

Figure 4. Surface-enhanced resonance Raman spectra of the cytochrome c Tyr67Phe mutant on a 4-PyS-modified silver electrode as a function of electrode potential: A) open circuit; B) -45 mV; C) 0 mV; D) +30 mV. Experimental conditions were the same as in Figure 3.

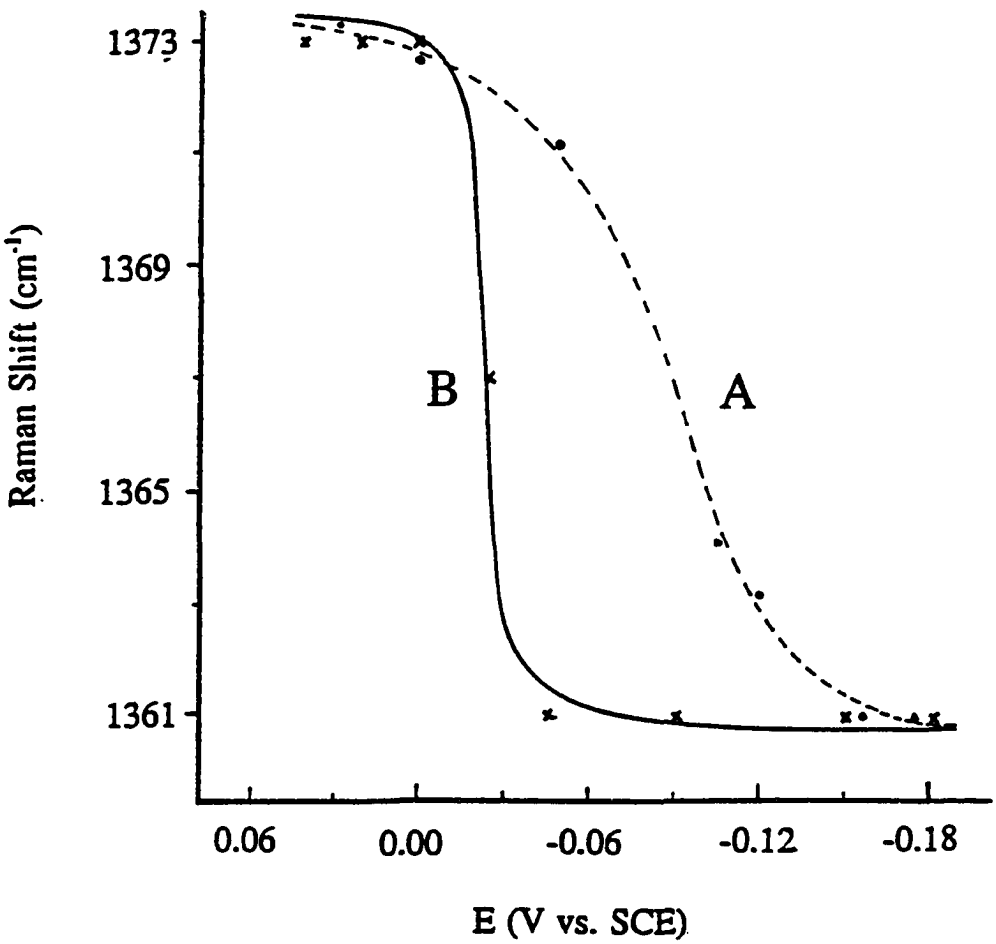

Figure 5. Potential dependence of the oxidation state marker band in the surface-enhanced resonance Raman spectrum of the Tyr67Phe mutant at (A) a bare silver electrode, (B) a 4-PyS-modified silver electrode.

Table 2: Fully oxidized and reduced potentials, potential ranges and estimated redox potentials of cyt.c mutants based upon the SERRS data.

protein	fully reduced potential (mv)	Fully oxidized potential (mV)	potential range (mV)	estimated redox potential (mV)
WT	-40	30	70	12
His26Val	-100	0	100	-5
Asp50Arg	-35	40	75	12
Tyr67Phe	-45	30	75	-22
Asn52Ile	-80	0	80	-30
Thr78Val	-70	20	90	-25

and that the estimated redox potentials in Table 2 are close to the redox potentials in Table 1 for corresponding mutants. Thus, the SERRS results agree well with the CV results. The potential ranges obtained from the SERRS results indicate that most of the cytochrome c mutants have similar electrochemical reversibility on the modified silver electrodes, except His26Val, which was discussed above.

Both the redox potentials of Table 1 and the estimated redox potentials of Table 2 show that the so-called "water mutants" have lower redox potentials than those of other cytochromes which means that the mutations increase the stability of the oxidized form of the protein. It was proposed [4] that mutations in these mutants, with loss of the internal water molecule, result in a configuration which resembles the oxidized conformation more closely than the reduced and this localized conformational change is likely to account for part of the shift in the redox potential. The mutation also affects the hydrogen-bonding of an interior water molecule and leads to a general increase in the hydrophobicity of the protein in the domain occupied by the mutated residues [15]. The water mutants examined in this study include the following: Tyr67Phe, Asn52Ile and Thr78Val. Figure 6 shows the diagram of the structure of cytochrome c in the region surrounding these mutations [23]. Residues on the 'left side' of heme plane are involved in maintaining a single water molecule in the hydrophobic interior of the protein, near the 'lower' edge of heme plane [24,25]. These include the invariant residues tyrosine 67 ('top left'), asparagine 52 ('bottom left') and threonine 78 ('lower front'). It was reported that all three of these "second tier" mutants, involving residues which interact with the heme ligands but are not themselves ligands, exhibit increased stability of the closed functional

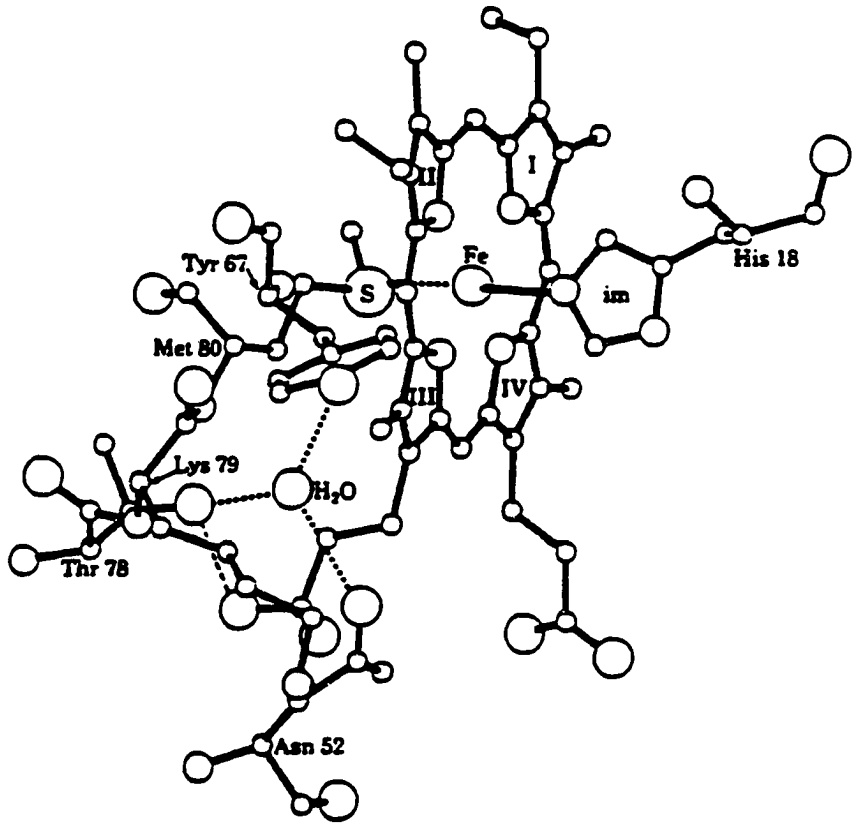

Figure 6. Diagram of the structure of cytochrome c in the region important to the water mutation studied here. Fe indicates the heme iron atom, im shows the imidazole side chain of histidine 18, and S is the sulfur atom of methionine 80. These are the axial ligands of the heme iron atom, from the "right" and the "left" sides of the protein molecule, respectively. I, II, III, and IV indicate the corresponding pyrrole rings of the heme. The amino acid residues are indicated in three-letter code placed near their α-carbon atoms. The molecule is viewed from the "front", defined as the area containing the solvent-accessible edge of the heme plane, containing pyrrole rings II and III. The heme plane is slightly titled to the left, so that the imidazole axial ligand on the right side has moved closer to the viewer than the sulfur axial ligand on the other side of the heme plane. H_2O marks the internal water molecule hydrogen-bonded (dotted lines) to the side chains of asparagine 52, tyrosine 67, and threonine 78 [17].

form of the heme crevice based upon measurement of the changes in the 695 nm absorption band of cytochrome (sensitive to the Fe-methionine bond) as a function of pH, heat and urea [10]. From the amino acid structure it can be seen that all of the three residues, tyrosine 67, asparagine 52 and threonine 78 are polar, but in the water mutants they are substituted by the non-polar residues phenylalanine, isoleucine and valine. Since three hydrogen bonds are required to hold the water molecule in the protein interior [26], the substitution of polar residues with non-polar residues in the water mutants eliminates one of these hydrogen bonds and leads to expulsion of the water molecule from its intramolecular position. This also liberates the asparagine 52 and threonine 78 side chains from their unfavorable internal position which may account for part of the increase in protein stability [23].

Resonance Raman spectroscopy of protein in the low frequency region can provide information about the interactions between amino acids and heme group. The resonance Raman spectra of cytochrome and Thr78Val and Tyr67Phe mutants in the low frequency region are shown in Figure 7 and Figure 8. Table 3 lists the resonance Raman frequencies and their normal mode assignments for wild type and the three water mutants of cytochrome c in the low frequency region. From the Table 3 it can be seen that there are considerable differences between wild-type cytochrome c and the water mutants. The γ_{22} band of Asn52Ile mutant decreases to a shoulder and a new band appears at 443 cm^{-1}. The ν_{26} and γ_{24} bands of Tyr67Phe mutant become shoulders and a new ν_{52} band is observed at 260 cm^{-1}. The greatest changes occur in the Thr78Val mutant. The γ_{13}, ν_{18}, ν_{34}, and ν_{53} bands become very weak. The ν_{26} band is shifted from 240 to 248 cm^{-1}. A new band γ_7

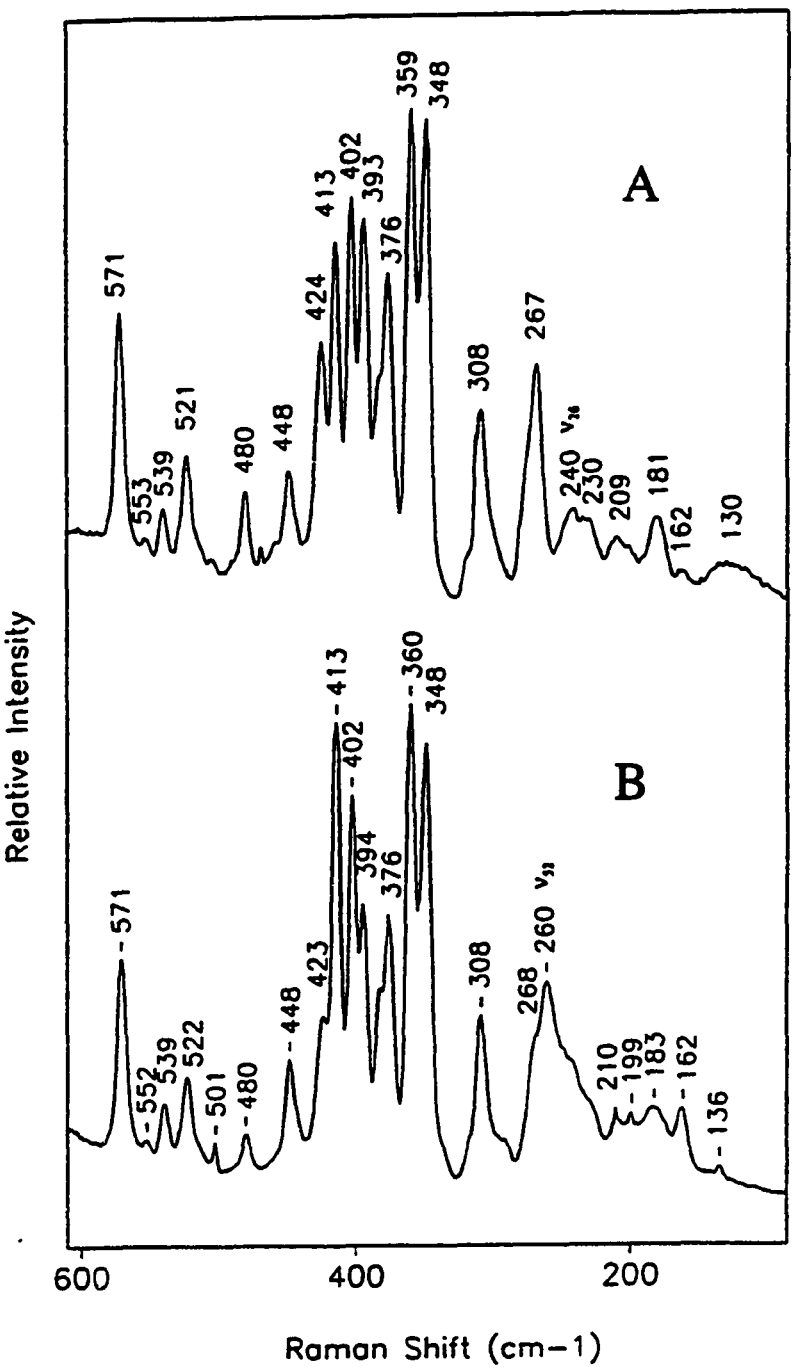

Figure 7. Resonance Raman spectra of 0.1 mM reduced cytochrome c (A) wild-type and (B) Tyr67Phe mutant at 77 K. Experimental conditions were the same as in Figure 3. The cytochromes were reduced by sodium dithionite.

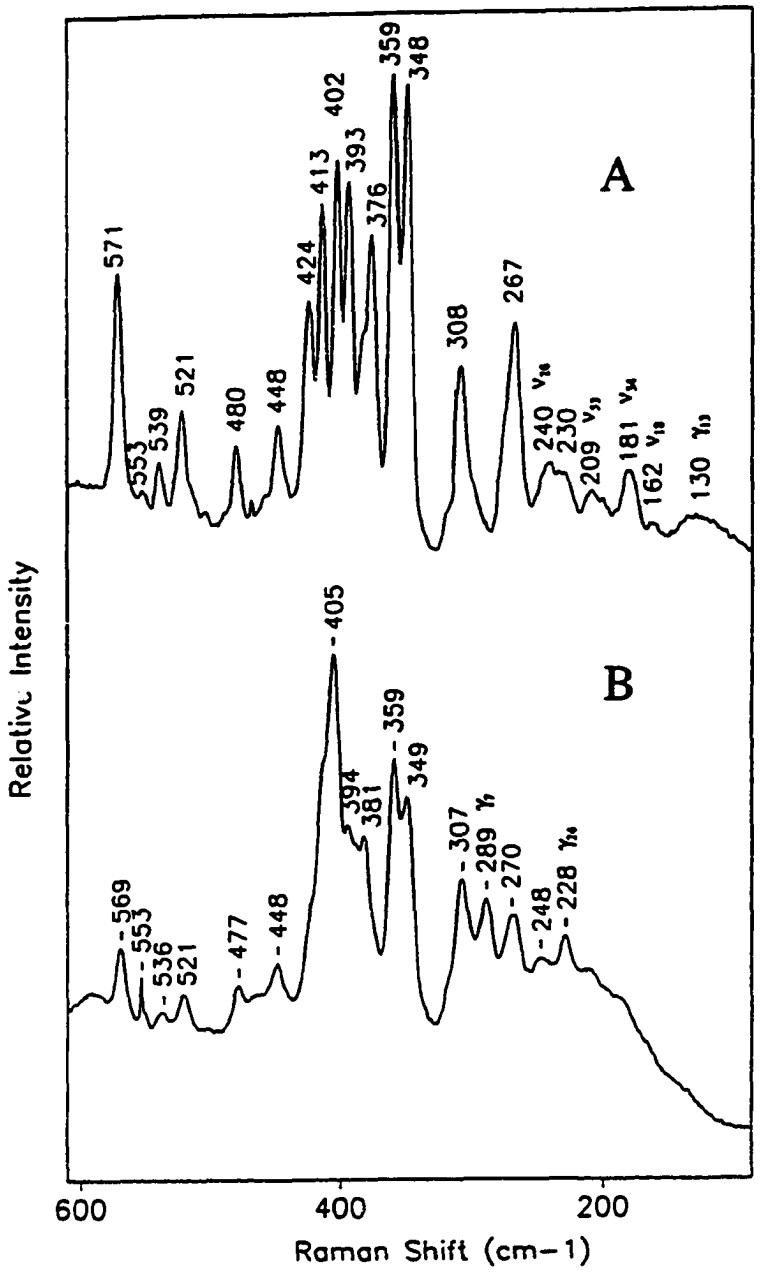

Figure 8. Resonance Raman spectra of 0.1 mM reduced cytochrome c (A) wild-type and (B) Thr78Val mutant at 77 K. Experimental conditions were the same as in Figure 7.

Table 3. Resonance Raman Frequencies and Their Normal Mode Assignments for Cytochrome c and The Water Mutants

Assignment	Local Coordinate	WT	Thr78Val	Tyr67Phe	Asn52Ile
γ_{21}	$(\text{pyr fold})_{sym}$	571	569	571	571
ν_{49}	δ(pyr rotat)	539	536	539	536
γ_{12}	pyr swivel	521	521	522	521
ν_{33}	δ(pyr rotat)	480	477	480	480
γ_{22}	pyr swivel	448	448	448	450 , 443
$\delta(C_\beta C_a C_b)$	$\delta(C_\beta C_a C_b)$	424, 413	424 , 413	423, 413	421, 415
$\delta(C_\beta C_a S)$	$\delta(C_\beta C_a S)$	402, 393	405, 394	402, 394	402, 394
$\delta(C_\beta C_c C_d)$	$\delta(C_\beta C_c C_d)$	381,376	381	381, 376	381, 372
ν_{50}	porph def	359	359	360	360
ν_8	porph def	348	349	348	347
ν_{51}	$\delta(C_\beta C_1)_{asym}$	308	307	308	310
ν_9	$\delta(C_\delta C_1)_{sym}$	267	270	268	271
γ_7	$\gamma(C_\alpha C_m)$		289		
ν_{52}	$\delta(C_\delta C_1)_{sym}$			260	
ν_{26}	$\delta(C_\delta C_1)_{sym}$	240	248	240	242
γ_{24}	$\gamma(C_\alpha C_m)$	230	228	230	228
ν_{53}	δ(pyr transl)	209		210	206
ν_{34}	$\delta(C_\delta C_1)_{sym}$	181		183	185
ν_{18}	porph def	162		161	161
γ_{13}	$\gamma(C_\alpha C_m)$	130		136	135

appears at 289 cm^{-1}. The $\delta(C_\beta C_c C_b)$ bands at 424 and 413 cm^{-1} decrease to shoulders. The $\delta(C_\beta C_c C_d)$ band at 376 cm^{-1} totally disappears. These differences indicate that the heme environment is strongly perturbed by the mutation. The side chain of threonine 78 forms a hydrogen bond both to an interior water molecule and the anterior propionyl side chain of the heme [24,25,27,28]. When threonine is replaced by a valine in cytochrome c, both hydrogen bonds are broken, and there is a dramatic change in the properties of the protein [10]. The hydrogen bond between the threonine 78 and the anterior propionyl side chain of the heme holds the anterior propionyl side chain in its position and makes the $C_\beta C_c C_d$ bond rigid. Breaking this hydrogen bond will liberate the anterior propionyl side chain of the heme and make the $C_\beta C_c C_d$ bond more flexible. Thus, the $\delta(C_\beta C_c C_d)$ band at 376 cm^{-1} disappears after the mutation.

Conclusion

Most of cytochrome c mutants examined in this study behave similarly with respect to electron transfer kinetics at electrodes because the mutations do not significantly change the protein surface structure. The water mutants Tyr67Phe, Thr78Val, and Asn52Ile have much lower redox potentials than those of other cytochromes indicating that the mutation increases the stability of the oxidized form proteins. The surface-enhanced resonance Raman results also indicate that the heme groups of the adsorbed cytochrome c and its mutants are in the six-coordinate low spin (6cLS) configuration at bis(4-pyridyl) disulfide-modified-silver electrodes, whereas mixtures of both 6cLS and five-coordinate high spin (5cHS) heme groups are present at the bare silver electrodes. The resonance Raman spectra of these mutants show considerable differences in the low frequency region as

compared to the wild-type cytochrome c which indicates that the heme environment is strongly perturbed by the mutation.

Acknowledgements

The authors are grateful for the financial support of the National Institute of Health (GM 35108).

References

1. J. E. Frew and H. A. O. Hill, *Eur. J. Biochem.* **1988,** 172, 261.
2. M. Tominaga, K. Hayashi and I. Taniguchi, *Anal. Sci.* **1992,** 8, 829.
3. F. A. Amstrong, H. A. O. Hill and N. J. Walton, *Acc. Chem. Res.* **1988,** 21, 407.
4. A. L. Burrows, L. Guo, H. H. O. Hill, G. McLendon and F. Sherman, *Eur. J. Biochem.* **1991,** 202, 543.
5. H. A. O. Hill and D. Whitford, *J. Electroanal. Chem.* **1987,** 235, 153.
6. S. Song, R. A. Clark, E. F. Bowden and M. J. Tarlov, *J. Phys. Chem.* **1993,** 97, 6564.
7. J. M. Cooper, K. R. Greenough and C. J. McNeil, *J. Electroanal. Chem.* **1993,** 347, 267.
8. X. Qu, T. Lu, S. Dong, C. Zhou and T. M. Cotton, *Bioelectrochem. and Bioenerg.*, **1994,** 34, 153.
9. Z. Fang, S. Imabayashi, T. Kakiuki and K. Niki, *J. Electroanal. Chem.* **1995,** 394, 149.

10. E. Margoliash, A. Schejter, T. I. Koshy, T. L. Luntz and A. E. Garber, in *Bioenergetics*, C. H. Kim and T. Ozawa, Eds., Plenum Press, New York, **1990.** 125-145.

11. A. J. Wand and D. L. Stefano, *Biochemistry*, **1989**, 28, 186.

12. Y. Feng, H. Roder, S. W. Englander, A. J. Wand and D. L. Stefano, *Biochemistry*, **1989**, 28, 195.

13. S. Fergruson-Miller, D. L. Brautigan and E. Margoliash, *J. Biol. Chem.*, **1978**, 253, 149.

14. E. Margolissh and H. R. Bosshard, *TIBS*, **1983**, 8, 316.

15. A. Schejter, T. I. Koshy. T. L. Luntz, R. Sanishvili, I. Vig and E. Margoliash, *Biochem. J.* **1994**, 302, 95.

16. H. Zhou, *J. Am. Chem. Soc.*, **1994**, 116, 10362.

17. S. Othman, A. L. Lirzin and A. Desbois, *Biochem.*, **1994**, 33, 15437.

18. N. Parthasarathi, C. Hansen, S. Yamaguchi and T. G. Spiro, *J Am. Chem. Soc.*, **1987**, 109, 3865.

19. T. M. Cotton, J. H. Kim and G. D. Chumanov, *J. Raman Spectrosc.* **1991**, 22, 742.

20. R. Picorel, R. E. Holt, R. Heald, T. M. Cotton and M. Seibert, *J. Am. Chem. Soc.*, **1991**, 113, 2839.

21. R. E. Dickerson, *Sci. Amer.* **1972**, April, 58

22. M. J. Eddowes, H. A. O. Hill and K. Uosaki, *J. Am. Chem. Soc.* **1979**, 101, 7113.

23. A. Schejter, T. L. Luntz, T. I. Koshy and E. Margoliash, *Biochem.* **1992,** 31, 8336.

24. T. Takano and R. E. Dickerson, *J. Mol. Biol.* **1981,** 153, 79.

25. T. Takano and R. E. Dickerson, *J. Mol. Biol.* **1981,** 153, 95.

26. A. A. Rashin, M. Iofin and B. Honig, *Biochem.* **1986,** 25, 3619.

27. H. Ochi, Y. Hata, N. Tanaka, M. Kakudo, T. Sakurai, S. Aihara and Y. Morita, *J. Mol. Biol.* **1983,** 116, 407.

28. G. V. Louie, W. L. B. Hutchinson and G. D. Brayer, *J. Mol. Biol.* **1989,** 199, 295.

GENERAL SUMMARY

Conventional electrochemical methods were coupled with structure sensitive techniques, resonance Raman and surface-enhanced resonance Raman spectroscopies to study the electrochemical behavior, electron transfer mechanisms and configuration of the cytochrome c at modified metal electrode surfaces. This combination provided structural as well as thermodynamic and kinetic information on the reaction of cytochrome c at an electrode/solution interface. Chapter 1 reevaluated two promoters, 2,2'-bipyridine and pyrazine which were studied by other research groups and a new electrode modification procedure was used. It was determined that both 2,2'-bipyridine and pyrazine act as promoters when self adsorbed over a sufficiently long dipping time. The promoter characteristics of these two molecules were studied and compared with those of 4,4'-bipyridine. The difference in their promoter behavior appears to result primarily from their different strengths of adsorption and not because electrodes modified with 2,2'-bipyridine or pyrazine are unsuitable for accelerating direct electron transfer reaction in cytochrome c.

In Chapter 2, it was found that molecules with one functional group, such as pyridine, carbazole and thiophene can also accelerate the electrochemical reaction of cytochrome c. The behavior of the different promoters with one functional group indicates that an important requirement for an effective promoter is that it interacts with the electrode surface. The crucial role of these three promoters may be to prevent denaturation and/or irreversible adsorption of cytochrome c or the deaminated and oligomeric components, which results in irreversible electrochemistry.

In Chapter 3, Quasi-reversible and direct electron transfer was observed between an iodide-modified gold electrode and cytochrome c. The results suggest that an electrostatic interaction between cytochrome c and the iodide-modified electrode surface plays an important role in the electrochemical response. Results obtained by surface enhanced resonance Raman scattering (SERRS) spectroscopy indicate that the heme group of the adsorbed cytochrome c is in the native low spin, six coordinate configuration at the iodide-modified Ag electrode, whereas at the bare Ag electrode a mixture of both low spin, six coordinate and high spin, five coordinate heme is present on the surface. A possible mechanism for the role of iodide as a promoter of cytochrome c electrochemistry is that the adsorbed iodide ions on the electrode surface change the surface charge of the electrode which leads to a favorable orientation of cytochrome c at the electrode surface for the electron transfer reactions.

In Chapter 4, several cytochrome c mutants have been characterized by CV, RR and SERRS in order to study how the mutations affect the redox potential, electron transfer kinetics and the stability of cytochrome c, and to understand more about the structure/function relationships in redox proteins. It was found that most of cytochrome c mutants behave similarly at electrodes in terms of electron transfer kinetics because the mutations did not significantly change the protein surface structure. The water mutants Tyr67Phe, Thr78Val, and Asn52Ile have much lower redox potentials than those of other cytochromes indicating that these mutations increase the stability of the proteins. The resonance Raman spectra of the water mutants show considerable differences as compared to the wild-type cytochrome c which indicates that the heme environment is strongly

perturbed by the mutation. The surface-enhanced resonance Raman results also indicate that the heme groups of the adsorbed cytochrome c and its mutants are in the six-coordinate low spin (6cLS) configuration at bis(4-pyridyl) disulfide-modified-silver electrodes, whereas mixtures of both 6cLS and five-coordinate high spin (5cHS) heme groups are present at the bare silver electrodes.

ACKNOWLEDGEMENTS

I would like take this opportunity to thank all those who have provided help in the course of my study at Iowa State University. I am specially grateful to my advisor, Dr. Therese M. Cotton, for her guidance, support, and encouragement throughout my graduate career. I also wish to thank Dr. Tianhong Lu for the time and effort he spent in order to help me with the SERRS experiments, and Dr. George Chumanov for his help with the laser and Raman instrumentation. I want to thank Professor Katsumi Niki for sharing his knowledge in this area. My sincere thanks also go to every member of the Cotton group: Al, Brian C., Brian G., Dale, Morgan, Kostia, Yuri, Zheng and Shuyu. I have enjoyed working with you guys and thank you for the great volleyball games.

Finally, special thanks go to my wife Jianping for her love and support throughout my graduate studies.