Redox Reactions of Cytochrome *c* on Self-assembled Monolayers of Mercaptobenzoic Acid and Mercaptohydrocinnamic Acid Adsorbed on Au (111) Electrodes

Shin-ichiro IMABAYASHI*, Takahiro MITA, Zhi Qiang FENG†, Minehiko IIDA, Katsumi NIKI† and Takashi KAKIUCHI

Received December 2, 1996 ; Accepted March 3, 1997

The redox reactions of cytochrome *c* (cyt *c*) adsorbed on self-assembled monolayers (SAMs) of 4-mercaptopbenzoic acid (4-MBA) and 4-mercaptophydrocinnamic acid (4-MHCA) are studied using cyclic voltammetry and ER spectroscopy. A monolayer of cyt *c* in a stable, electroactive state is formed on close-packed monolayers of both aromatic thiols. The redox potentials of cyt *c* are 35 mV for 4-MBA and 20 mV vs. Ag/AgCl for 4-MHCA. The electron transfer (ET) rate constants, k_{et} , of cyt *c* at pH 7 are determined from the frequency dependence of the ER response to be 1800 s⁻¹ for 4-MBA system and 3900 s⁻¹ for 4-MHCA. The k_{et} of adsorbed cyt *c* tends to be larger on the SAMs of both aromatic thiols than on the SAM of 6-mercaptophexanoic acid having a similar thickness, suggesting that the difference in the SAM structure is reflected to the k_{et} of cyt *c*.

1 INTRODUCTION

The electrochemical characterization of strongly adsorbed or covalently attached electroactive species at self-assembled monolayer(SAM)s of alkanethiols and dialkyl disulfides have been carried out to elucidate fundamental aspects of electron transfer (ET) reactions ¹⁾. Chidsey et al.²⁾ and Finklea et al.³⁾ have investigated the ET reactions of ferrocene and pentaammine(pyridine)ruthenium complex covalently attached at SAM surfaces and demonstrated that the SAMs can be used to systematically study the dependence of ET rates on distance, medium, and spacer structure. Bowden et al. have applied this approach to study the ET of cytochrome *c* (cyt *c*) immobilized on carboxylic acid-terminated SAMs ⁴⁾. They showed that cyt *c* is stably adsorbed on these SAMs and cyt *c* / SAM / gold systems are useful for characterizing the ET reactions of proteins.

In intramolecular ET reactions in linked electron donor-acceptor compounds, the ET rate through aromatic or polyene linkages is larger than that through aliphatic linkages, because the former linkages provide better electronic coupling ⁵⁾. A number of modified electrodes with not only aliphatic thiols but aromatic thiols have been used for studying ET reactions between electrodes and redox proteins ^{6, 7)}. An understanding of the influence of the SAM structure on the ET between electrodes and proteins immobilized on a SAM surface is significant for the design and the preparation of such supra-biomolecular systems as intelligent biosensors and biomolecular devices.

In the present paper, we have measured the apparent ET rate constant, k_{et} , of cyt *c* adsorbed on SAM surfaces of 4-mercaptopbenzoic acid (4-MBA) and 4-mercaptophydrocinnamic acid (4-MHCA) by potential-modulated UV-vis reflectance (ER) spectroscopy. The obtained k_{et} values were compared with those of cyt *c* adsorbed on SAMs composed of COOH-terminated alkanethiols having a series of alkyl chain length ^{8, 9)}. The amount of adsorbed thiols

Department of Physical Chemistry, Yokohama National University (Tokiwadai, Hodogaya-ku, Yokohama 240, Japan)

* Department of Chemistry, Iowa State University (Ames, Iowa 50011, USA)

Key words: Cyt *c*, Self-assembled monolayer, Mercaptobenzoic acid, ER spectroscopy.

on gold electrodes and the pK_a value of the COOH group of the SAM were also electrochemically determined.

2 EXPERIMENTAL

2.1 Reagents

4-MBA and 4-MHCA were purchased from Toronto Research Chemicals Inc. and Tokyo Kasei Co., respectively, and were used without purification. Cyt *c* (Sigma Type VI, horse heart) was purified by a cation exchange column (Whatman, CM-52). Water was distilled and purified with a Milli-Q system (Millipore Co.). All other chemicals were of reagent grade and used without purification.

2.2 Sample preparation

Au substrates prepared by vapor deposition of Au (99.99% purity)¹⁰ were immersed in 1 mM ethanolic solution of the aromatic thiols for 3 hours to form a thiol monolayer. The substrates were then taken out, rinsed with ethanol, and dried in air. A small amount of 30 mM sodium phosphate buffer (pH = 7) containing 200 μ M cyt *c* was added on the top of the thiol-modified Au substrate and left for 30 min, followed by several rinsings of the substrate with the buffer to remove a loosely adsorbed cyt *c*. The peak potential of the redox wave and the amount of adsorbed cyt *c* evaluated from the area of cyclic voltammograms were independent on the adsorption time of cyt *c* up to 24 h.

2.3 Electrochemical measurements

The reductive desorption of the aromatic thiols from a gold substrate was measured as described previously¹⁰. The pK_a value of the COOH group of the SAM was estimated from the pH dependence of the double-layer capacitance (C_d) at the solution/electrode interface¹¹.

The instrumentation for ER spectroscopy¹² and the cell configuration for evaporated gold substrates used in cyclic voltammetry, ac impedance measurements and ER spectroscopy⁹ were described elsewhere. The ET rates of cyt *c* adsorbed on SAMs were evaluated as described previously¹³.

A Ag/AgCl electrode in saturated KCl solution was used as a reference electrode. All measurements were performed at 25 ± 2 °C after the solution was deaerated with Ar for 20–30 min.

3 RESULTS AND DISCUSSION

The peak potentials for the reductive desorption, E_p , of 4-MBA and 4-MHCA in 0.5 M KOH were -0.56 V and -0.63 V, respectively, which are 230–300 mV more positive than those of COOH-terminated alkanethiols with a similar molecular length^{10, 14}. A probable explanation for the difference in E_p between 4-MBA and 4-MHCA is that the attractive intermolecular interaction between ethylene chains of 4-MHCA stabilizes the monolayer and leads to a more negative E_p value as is the case of alkanethiols¹⁰. The amounts of adsorbed thiol for both 4-MBA and 4-MHCA estimated from the cyclic voltammograms for the reductive desorption accord with those for alkanethiol SAMs, indicating that both aromatic thiols form close-packed monolayers on Au(111) surface¹⁵.

In the pK_a measurements of COOH group of the SAM, the solution pH was initially changed from 2 to 12, followed by a reverse titration to pH 2. Figure 1 shows C_d vs. solution pH plots at $E_{dc} = 0$ V for both thiols. In both acid and alkaline direction titrations, the C_d changed in the range of pH 6–10, corresponding to the protonation-deprotonation of COOH. The hysteresis in C_d vs. pH plots was observed in all titration cycles. Assuming that the pK_a is a pH value at the mid-

Fig. 1 C_d vs. solution pH plots for 4-MBA (○, ●) and 4-MHCA (□, ■) adsorbed Au(111) electrodes at $E_{dc} = 0$ V, $\Delta E_{ac} = 7$ mV, $f = 40$ Hz and electrode area = 0.79 cm^2 . The pH was changed by adding dilute HClO_4 (○, □) or dilute NaOH (●, ■) to 0.1M NaClO_4 .

point of the C_d variation, the average pK_a s in both direction titrations are estimated to be 7.6 for 4-MBA and 8.2 for 4-MHCA. The electron-withdrawing effect of the SH group at the para position of benzene ring¹⁶⁾ probably cause the slightly smaller pK_a value for 4-MBA. The large shift in the pK_a value of COOH group at those SAM surfaces against that in solution is mainly attributable to the electrostatic repulsion among deprotonated COOH (COO^-) groups¹¹⁾. The pK_a values indicate that the fraction of COO^- at these SAM surfaces is below 25% at pH 7.

Figure 2 shows cyclic voltammograms for cyt *c* adsorbed on SAMs of 4-MBA (a) and 4-MHCA (b) in a 10 mM phosphate buffer at pH 7. The peak potentials of the redox reaction of cyt *c* were 35 ± 10 mV for 4-MBA and 20 ± 10 mV for 4-MHCA. These potentials are 20–40 mV more positive than that for cyt *c* in the solution phase¹⁷⁾. The peak current was proportional to the sweep rate, as expected for the redox reaction of adsorbed species. The amount of adsorbed cyt *c* estimated from the area of voltammetric peak was 1.4×10^{-11} mol/cm² for both systems, which is close to that of the full coverage, 1.5×10^{-11}

Fig. 2 Cyclic voltammograms for cyt *c* adsorbed on SAMs of 4-MBA (a) and 4-MHCA (b) in 10 mM phosphate buffer at pH 7: initial potential, 0.3 V; sweep rate, (1) 20 mV / s, (2) 50 mV / s, (3) 100 mV / s, and (4) 200 mV / s; electrode area, 3.0 cm².

mol/cm², estimated from the molecular size of cyt *c*: $30 \times 34 \times 34$ Å¹⁸⁾. The voltammetric response of cyt *c* was stable for at least 3 hours at room temperature and also unchanged under the continuous potential cyclings between +0.3 V and -0.2 V. Since the fraction of COO^- group at the SAM surfaces is low at pH 7, it is suggested that not electrostatic interaction between lysines on cyt *c* surface and COO^- groups but hydrogen bonding between lysines and COOH groups plays a major part in the stable adsorption of cyt *c* on the SAMs.

Figure 3 shows ER spectra for cyt *c* / 4-MBA / Au system at pH 7. The α , β , and Soret bands appeared in the ER spectra, which agree well with the difference absorption spectrum between reduced and oxidized states of cyt *c*¹⁹⁾. Similar ER spectra were obtained for cyt *c* / 4-MHCA / Au system.

ER voltammograms monitored at 418 nm for both cyt *c* / 4-MBA / Au and cyt *c* / 4-MHCA / Au systems at pH 7 are shown in Fig. 4. The peak potentials agree with those of the cyclic voltammograms in both systems. The ratio of the height of the real peak to imaginary one was greater for cyt *c* / 4-MHCA / Au than for cyt *c* / 4-MBA / Au, indicating that the k_{et} of cyt *c* is larger in the former. The k_{et} of cyt *c* evaluated from the frequency dependence of the ER response were 1800 s⁻¹ for cyt *c* / 4-MBA / Au and 3900 s⁻¹ for cyt *c* / 4-MHCA / Au. If both aromatic thiols adsorb

Fig. 3 ER spectra for cyt *c* / 4-MBA / Au in the same solution as in Fig. 2: $E_{dc} = 50$ mV, $f = 8$ Hz and $\Delta E_{ac} = 50$ mV.

Fig. 4 ER voltammograms for cyt *c* / 4-MBA / Au (solid) and cyt *c* / 4-MHCA / Au (broken) in the same solution as in Fig. 2: $\lambda = 418$ nm, $f = 8$ Hz, $\Delta E_{ac} = 50$ mV and $v = -2$ mV/s.

perpendicularly to the electrode surface, the thickness of the SAM would be 9.5 Å for 4-MBA and 10.5 Å for 4-MHCA. However, the k_{et} of the latter system is about 2 times larger than that of the former. This relationship between the k_{et} and the thickness of SAM is distinct from that obtained for cyt *c* adsorbed on COOH-terminated alkanethiol SAMs, in which the k_{et} becomes smaller with increasing the chain length of thiol⁹⁾. This suggests that the ET rate is not a simple function of the distance between the adsorbed cyt *c* and the electrode surface; the ET pathway and the orientation of adsorbed cyt *c* in cyt *c* / 4-MHCA / Au is different from those in cyt *c* / 4-MBA / Au.

The k_{et} s of cyt *c* adsorbed on the aromatic SAMs are larger than 1100 s⁻¹ for cyt *c* adsorbed on the SAM of 6-mercaptopentanoic acid having a similar thickness under the same experimental conditions,⁹⁾ suggesting that the SAM containing aromatic groups provides a larger k_{et} for the ET between the adsorbed cyt *c* and the electrode surface, as do aromatic linkages for the ET in linked donor-acceptor compounds⁶⁾.

We gratefully acknowledge to the NEDO for financial support. This work was supported in part by Grant-in-Aid for Encouragement of Young

Scientists (No. 06855096 for S.I.) from the Ministry of Education, Science and Culture, Japan. S.I. also gratefully acknowledges support of this work by the Saneyoshi Scholarship Foundation.

References

- 1) L. H. Dubois and R. G. Nuzzo, *Annu. Rev. Phys. Chem.*, **43**, 437 (1992), and references therein.
- 2) C. E. D. Chidsey, *Science*, **251**, 919 (1991).
- 3) H. O. Finklea and D. D. Hanshaw, *J. Am. Chem. Soc.*, **114**, 3173 (1992).
- 4) S. Song, R. A. Clark, E. F. Bowden and M. J. Tarlov, *J. Phys. Chem.*, **97**, 6564 (1993).
- 5) B. E. Bowler, A. L. Raphael, and H. B. Gray, *Prog. Inorg. Chem.*, **38**, 259 (1990).
- 6) P. M. Allen, H. A. O. Hill, and N. J. Walton, *J. Electroanal. Chem.*, **178**, 69 (1984).
- 7) A. M. Bond, *Inorg. Chim. Acta*, **226**, 293 (1994).
- 8) Z. Q. Feng, S. Imabayashi, T. Kakiuchi and K. Niki, *J. Electroanal. Chem.*, **349**, 149 (1995).
- 9) Z. Q. Feng, S. Imabayashi, T. Kakiuchi and K. Niki, submitted to *J. Chem. Soc. Faraday Trans.*
- 10) S. Imabayashi, M. Iida, D. Hobara, Z. Q. Feng, T. Kakiuchi, and K. Niki, *J. Electroanal. Chem. Soc.*, in press.
- 11) T. Kakiuchi, M. Iida, N. Gon, and S. Imabayashi, to be published.
- 12) T. Sagara, S. Igarashi, H. Sato, and K. Niki, *Langmuir*, **7**, 1005 (1991).
- 13) Z. Q. Feng, T. Sagara and K. Niki, *Anal. Chem.*, **67**, 3564 (1995).
- 14) C. A. Widrig, C. Chung, and M. D. Porter, *J. Electroanal. Chem.*, **310**, 335 (1992).
- 15) S.-C. Chang, I. Chao, and Y.-T. Tao, *J. Am. Chem. Soc.*, **116**, 6792 (1994).
- 16) N. B. Chapman, J. Shorter, eds., *Correlation Analysis in Chemistry*, Plenum Press, 1978, pp.439-540.
- 17) C. Hinnen, C. Nguyen van Huong, A. Rousseau and J. P. Dalbera, *J. Electroanal. Chem.*, **95**, 131 (1979).
- 18) R. E. Dickerson, T. Takano, D. Eisenberg, O. B. Kallai, L. Samson, A. Cooper, and E. Margoliash, *J. Biol. Chem.*, **246**, 1511 (1971).
- 19) T. Sagara, H. Murakami, S. Igarashi, H. Sato and K. Niki, *Langmuir*, **7**, 3190 (1991).