Unexpected retention of electrostatically adsorbed cytochrome *c* in high ionic strength solutions

Cory M. DiCarlo and David L. Compton*

Received (in Cambridge, UK) 21st September 2004, Accepted 4th November 2004
First published as an Advance Article on the web 6th December 2004
DOI: 10.1039/b414441d

Redox inactivation, but not removal, of electrostatically adsorbed cytochrome *c* (cyt-c) on an alkanethiol modified gold surface was observed after exposure of the electrode to 1.0 M aqueous NaCl, $NaClO_4$, KCl, or $KClO_4$ solutions.

Immobilization of proteins onto various modified electrode surfaces has provided the additional opportunity to investigate many protein properties such as electron transfer kinetics[1–3] and redox thermodynamics.[3–5] In addition, immobilized protein systems have been increasingly investigated as platforms for bioelectrocatalytic processes and sensor development.[6,7] Due to several factors, including the relative ease of the immobilization process, cyt-c is one of the most studied of the protein–alkanethiol systems.[3] In most cases, the cyt-c is either electrostatically adsorbed or covalently immobilized to an alkanethiol modified electrode surface. The success of that immobilization is frequently determined through electrochemical means such as cyclic voltammetry (CV). Covalent immobilization requires a rinsing step involving a high ionic strength solution, which is intended to remove electrostatically adsorbed protein and leave only covalently attached protein on the electrode surface. In this study, however, electrostatically adsorbed cyt-c is found to be retained after rinsing with high ionic strength solutions. Of concern is the observation of a temporary inactivation of the cyt-c redox signal after exposure to high ionic strength solutions, as measured by CV, which may be misconstrued as the desorption of cyt-c from the electrode.

As electrostatically adsorbed protein has different properties from covalently attached protein, a masked retention of electrostatic adsorbed protein with covalent protein could cause errors in subsequently measured data and the interpretation of experiments. This finding therefore represents an important consideration for researchers using this and similar systems.

Electrostatically adsorbed cyt-c electrodes were created by modifying a gold wire electrode with a self-assembled monolayer (SAM) *via* exposure to a 1 : 1 mixture of 11-mercapto-1-undecanol (11-MU) and 11-mercaptoundecanoic acid (11-MUA) for 24 h and then exposing the modified electrode to a 30 μM solution of horse heart cyt-c for 3 h.[6] Mixed SAMs were used because they result in improved electron transfer to the immobilized cyt-c in aqueous buffers.[6] A redox couple was then measured using CV in 10 mM phosphate buffer solution (P_i), pH 7.0, with an $E^{\circ\prime} = -17 \pm 3$ mV (*vs.* Ag/AgCl reference electrode) and a peak separation of 13 ± 3 mV. This redox couple has been previously assigned to the Fe(II)/Fe(III) redox process of cyt-c electrostatically adsorbed onto the SAM used in this study. The coverage of electroactive cyt-c ($\Gamma^{\circ}_{\text{cyt-c}}$) based on the cathodic peak currents obtained by CV is 6.2 ± 0.8 pmol/cm^2. Good agreement exists between the average value of $E^{\circ\prime}$ obtained here and the average value obtained by Ge and Lisdat[6] of -21 ± 2 mV for the same protein/electrode modification system. The small peak separation and the absence of cyt-c in the solution used during CV measurement indicate that cyt-c is adsorbed onto the electrode surface.

Cyt-c modified electrodes were stirred in solutions of up to 1.0 M NaCl in P_i for 10 s, rinsed well with deionized H_2O, and then returned to P_i solution alone for measurement. Immediately after exposure, the cyt-c redox signal was no longer present on CVs. Contrary to expectations, subsequent voltammograms recorded the return of the cyt-c redox signal over time with no additional exposure to cyt-c solution, Fig. 1. The cyt-c redox charge was then found to return to near the original, non-exposed level. The rate of return of charge for the NaCl exposed cyt-c is shown in Fig. 2. In general, the redox charge was found to return to a steady value within 31 ± 14 minutes ($n = 3$ electrodes).

Additional salts, $NaClO_4$, KCl, and $KClO_4$ were investigated to determine if ionic radius would be a factor in the ability to recover cyt-c signal after loss. However, all three additional salts displayed the same magnitude of loss followed by the same rate of recovery, within the level of error for the measurements.

Cyt c has been shown to interact with the hydrophobic regions of phospholipids.[8] To explore the possibility that a hydrophobic

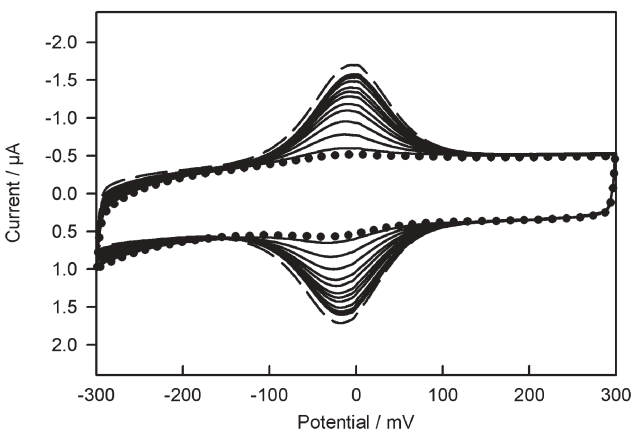

Fig. 1 Cyclic voltammograms showing return of absorbed cyt-c redox signal after exposure to 1.0 M NaCl. Pre-exposure signal (- - -), immediately after NaCl exposure and return to P_i (···), and signal increasing with time (—).

*comptond@ncaur.usda.gov

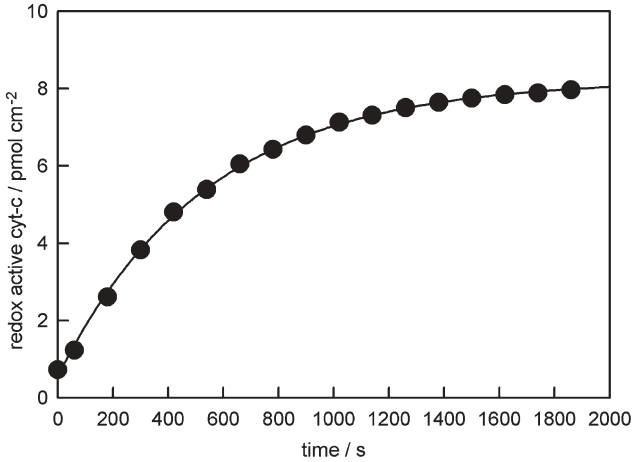

Fig. 2 Increase in cyt-c redox charge as a function of time after 1.0 M NaCl exposure.

region of cyt-c may be helping to anchor the protein to the SAM,[9] the electrodes were exposed to low concentrations of ethanol or methanol in conjunction with aqueous salt solutions. Previous studies by Borsari *et al.*[10] have examined the redox thermodynamics of bovine heart cyt-c in mixed water–organic solutions. In these studies it was found that methanol and ethanol had a negligible effect on the protein while using between 0–20% solutions of the solvents in conjunction with aqueous $NaClO_4$ solution. Beyond this range, the thermodynamic properties of the Fe(II)/(III) redox couple begin to change, indicating a change in the structure of the heme environment. Not wanting to complicate interpretation of the redox signal loss, only solutions at 10 and 20% organic content were examined. The cyt-c redox signal was unaltered in P_i containing 10 and 20% ethanol or methanol. In 1.0 M salt solutions containing 10 and 20% ethanol or methanol, the cyt-c signal followed the same profile of loss and slow recovery after reincubation in P_i as did the solutions containing inorganic salts alone. These results suggest that the retention of cyt-c on the SAM surface in the presence of 1.0 M salts is not due to hydrophobic interactions.

The reversible loss of redox signal without the apparent desorption of the cyt-c from the 11-MU/11-MUA SAM modified electrodes was surprising for two reasons. First, the protein has been previously described as being desorbed in the presence of 1.0 M KCl from the more electronegative 11-MUA SAMs.[11] Our investigation of the 11-MUA system found differing results. As found in the previous studies, exposure of the 11-MUA/cyt-c electrode to 1.0 M KCl resulted in loss of the cyt-c redox signal; however, in our experiments the redox signal was recovered by incubating the electrode in P_i containing no cyt-c. This indicates that desorption of the protein from the electrode did not occur. This result differs from that of Clark and Bowden which suggested that incubation in cyt-c solution was required for the recovery of cyt-c redox signal. Our experiments indicate instead that cyt-c is redox inactive but not desorbed from the 11-MUA or mixed 11-MUA:11-MU SAMs when exposed to 1.0 M salt solutions.

Secondly, although Qureshi *et al.*[4] have shown that denaturation of cyt-c begins to occur in the presence of simple inorganic salts such as LiCl and $CaCl_2$, the first transition in structure occurs at higher concentrations (> 1.0 M). In the present study, the complete (but temporary) loss of signal was observed even at concentrations of 0.20 M salt. The loss of redox signal at low salt concentrations, where previous studies have indicated that no or very minor structural change in the protein has occurred, indicates that denaturation of the protein is most likely not responsible for the redox signal loss. Surprisingly, the two most common interpretations for the loss of the electrostatically adsorbed cyt-c redox signal when exposed to high ionic strength aqueous solutions, desorption and denaturation, appear to be inaccurate.

The interaction between the SAM surface and the protein that allows for efficient redox activity, such as specific contact points between the protein and the SAM or a specific protein orientation, would be expected to be stabilized with covalent immobilization. Indeed, when cyt-c is covalently attached to the mixed SAM through established methods,[6] exposure to all four inorganic salts resulted in no loss of redox current.[12] Thus, even though the high ionic strength solutions do not result in the desorption or denaturation of the protein, the ions must interfere with the protein–SAM interface in some way to interrupt electron transfer. It is likely that the cations in the high ionic strength salt solutions interact with the electronegative SAM surface and significantly change the SAM surface charge distribution. This charge redistribution disrupts the electron transfer from the SAM to the electrostatically adsorbed cyt-c. It is possible that this charge redistribution allows the cyt-c molecules to reorient on the SAM into electrochemically inactive orientations without desorbing from the SAM surface. The slow return of the cyt-c redox signal is consistent with this view. The cations may desorb from the SAM in the P_i causing the redistribution of the SAM surface charge to its original state. The slow recovery of the redox signal may be caused by the slow reorientation of the cyt-c molecules to their initial electroactive states.

In conclusion, four inorganic salts, NaCl, $NaClO_4$, KCl, and $KClO_4$ were found to cause reversible loss of the observed redox signal of cyt-c electrostatically adsorbed onto a mixed 11-MU:11-MUA monolayer. The return of the cyt-c redox signal when the electrode is incubated in P_i containing no cyt-c indicates that the protein is not desorbed from the electrode when the signal is lost, but rather that the salt is interfering with the interfacial charge transfer process.

The lack of signal loss when covalently attached cyt-c is exposed to salt solution, as well as previous studies showing significant denaturation of cyt-c occurring only at concentrations higher than those used in this study, signifies that protein structural change is also an unlikely explanation for the temporary redox signal loss. As desorption and structural change appear unlikely, a loss of optimum orientation and interaction points due to charge redistribution of the SAM surface is suggested as the cause of the transitory CV redox activity loss.

Further studies, including quartz crystal microbalance with dissipative mechanism (QCM-D) and atomic force microscopy (AFM), are intended to confirm the physical retention of the protein on the electrode surface during redox inactivity. It is hoped that these methods will help clarify the nature of the redox-inactive form of the protein on the electrode surface.

Careful consideration must be taken with electrostatically immobilized cyt-c on alkanethiol modified electrodes to avoid

mistaking the inactivation of the cyt-c redox signal in high ionic strength solutions for desorption of the protein from the electrode.

Cory M. DiCarlo and David L. Compton*
New Crops and Processing Technology Research Unit, United States Department of Agriculture,† Agricultural Research Service, National Center for Agricultural Utilization Research, 1815 N. University St., Peoria, IL 61604, USA. E-mail: comptond@ncaur.usda.gov; Fax: 309-681-6524; Tel: 309-681-6321

Notes and references

1 L. J. C. Jeuken, *Biochim. Biophys. Acta*, 2003, **1604**, 67–76.
2 D. H. Waldeck, J. Wei, H. Yamamoto and H. Liu, *Langmuir*, 2003, **19**, 2378–2387.
3 M. Fedurco, *Coord. Chem. Rev.*, 2000, **209**, 263–331.
4 S. H. Qureshi, B. Moza, Y. Sushma and F. Ahmad, *Biochemistry*, 2003, **42**, 1684–1695.
5 G. Gavioli, M. Borsari, M. Cannio, A. Ranieri and G. Volponi, *J. Electroanal. Chem.*, 2004, **564**, 45–52.
6 B. Ge and F. Lisdat, *Anal. Chim. Acta*, 2002, **454**, 53–64.
7 K. V. Gobi and F. Nitzutani, *J. Electroanal. Chem.*, 2000, **484**, 172–181.
8 P. K. J. Kinnunen, A. Kõiv, A. Lehtonen, M. Rytömaa and P. Mustonen, *Chem. Phys. Lipids*, 1994, **73**, 181–207.
9 S. Oellerich, S. Lecomte, M. Paternostre, T. Heimburg and P. Hildebrandt, *J. Phys. Chem. B*, 2004, **108**, 3871–3878.
10 M. Borsari, M. Bellei, C. Tavagnacco, S. Peressini, D. Millo and G. Costa, *Inorg. Chim. Acta*, 2003, **349**, 182–188.
11 R. A. Clark and E. F. Bowden, *Langmuir*, 1997, **13**, 559–595.
12 C. M. DiCarlo, D. L. Compton, K. O. Evans and J. A. Laszlo, *Bioelectrochemistry*, 2004, submitted.

† Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may be suitable.

