# Electron Transfer and Ligand Binding to Cytochrome *c′* Immobilized on Self-Assembled Monolayers

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We have successfully immobilized *Allochromatium vinosum* cytochrome *c′* on carboxylic acid-terminated thiol monolayers on gold and have investigated its electron-transfer and ligand binding properties. Immobilization could only be achieved for pH's ranging from 3.5 to 5.5, reflecting the fact that the protein is only sufficiently positively charged below pH 5.5 (pI = 4.9). Upon immobilization, the protein retains a near-native conformation, as is suggested by the observed potential of 85 mV vs SHE for the heme $Fe^{III}/Fe^{II}$ transition, which is close to the value of 60 mV reported in solution. The electron-transfer rate to the immobilized protein depends on the length of the thiol spacer, displaying distance-dependent electron tunneling for long thiols and distance-independent protein reorganization for short thiols. The unique CO-induced dimer-to-monomer transition observed for cytochrome *c′* in solution also seems to occur for immobilized cytochrome *c′*. Upon saturation with CO, a new anodic peak corresponding to the oxidation of an $Fe^{II}-CO$ adduct is observed. CO binding is accompanied by a significant decrease in protein coverage, which could be due to weaker electrostatic interactions between the self-assembled monolayer and cytochrome *c′* in its monomeric form as compared to those in its dimeric form. The observed CO binding rate of 24 $M^{-1}\ s^{-1}$ is slightly slower than the binding rate in solution (48 $M^{-1}\ s^{-1}$), which could be due to electrostatic protein−electrode interactions or could be the result of protein crowding on the surface. This study shows that the use of carboxyl acid-terminated thiol monolayers as a protein friendly method to immobilize redox proteins on gold electrodes is not restricted to cytochrome *c*, but can also be used for other proteins such as cytochrome *c′*.

## Introduction

Over the last two decades, the immobilization of redox proteins on conducting surfaces has been actively pursued. Protein immobilization on electrodes allows one to probe electron-transfer mechanisms in proteins with electrochemical techniques.[1] From a more practical point of view, electrodes with immobilized redox enzymes can potentially be employed as biosensors[2,3] or as a new approach in biocatalysis, in which expensive electron donors and enzyme−product separation steps are no longer necessary.[4] A wide variety of methods using covalent, electrostatic, or hydrophobic interactions have been employed to immobilize redox proteins on both metal surfaces such as gold and silver as well as carbon surfaces such as pyrolitic graphite and glassy carbon.[5]

An important question is to what extent immobilization of a protein affects its properties. Detailed knowledge about the interactions between proteins and electrodes and the way electron transfer occurs between them is still lacking for most immobilized proteins. In some cases, the proteins have been shown to retain a near-native conformation as reflected in its enzymatic activity,[6−8] but in other systems significant conformational changes[9−11] or even cofactor release from the protein were observed after immobilization.[12,13]

Probably the best characterized immobilized protein system is horse heart cytochrome *c* on gold and silver, either bare or covered with ionic or hydrophobic self-assembled monolayers (SAM).[14−16] Surface-enhanced resonance Raman spectroscopy (SERRS)[17] and surface-enhanced infrared difference absorption spectroscopy (SEIDAS)[18] have shown that cytochrome *c* undergoes significant conformational changes on bare metal electrodes,[9,11] whereas it retains a near-native conformation on carboxylic acid-terminated SAMs.[17] SEIDAS[18] and protein engineering[19,20] studies have been used to identify the residues on cytochrome *c* that bind to the negatively charged carboxyl groups of the self-assembled monolayer. Additionally, detailed

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electron-transfer rate studies have been presented for SAMs of different lengths, showing rate limitation by electron tunneling and protein reorganization.[21−25] Although immobilization via hydrophobic interactions on SAMs of alkanethiols has also been shown to be suitable for certain proteins (azurin),[26,27] the studies on cytochrome *c* suggest that electrostatic immobilization is a relatively mild method that enables direct electron transfer between protein and electrode, but does not seem to significantly affect its structure and properties. Hence, it could be a suitable method for protein immobilization in general.

In this study, we investigate whether electrostatic immobilization on carboxylic acid-terminated SAMs can be extended to a more complex protein system. For this purpose, the protein cytochrome *c*′ from *Allochromatium vinosum* was chosen. This particular cytochrome *c*′ displays a unique reversible dimer-to-monomer transition upon binding of NO, CO, and $CN^-$.[28] This monomerization has been attributed to the displacement of Tyr16 from the distal site upon ligand binding, which results in a conformational change in one of the α-helices present at the dimer interface.[29] This cytochrome *c*′ thus provides an attractive model system to understand how binding of small diatomic ligands can induce large conformational changes.[30] Similar ligand-induced conformational changes are observed in heme-based sensor proteins such as guanylate cyclase (NO), CooA (CO), and FixL ($O_2$).[31−34] Here, we show that cytochrome *c*′ immobilized on carboxylic acid-terminated SAMs displays fast electron transfer between the protein and the electrode and discuss the orientation of the protein on the electrode. To test whether the ligand-induced conformational changes are impeded by the immobilization, the influence of CO and NO binding on the immobilized cytochrome *c*′ was studied.

## Experimental Procedures

**Materials.** Cytochrome *c*′ (*Allochromatium vinosum*) was expressed and purified as described previously.[35] Horse heart cytochrome *c* (95%, Fluka), 3-mercaptopropionic acid (99%, Aldrich) (MPA), 4-mercaptobutyric acid (95%, Pfaltz & Bauer) (MBA), 6-mercaptohexanoic acid (97%, Dojindo) (MHA), 8-mercaptooctanoic acid (97%, Dojindo) (MOA), 11-mercaptoundecanoic acid (95%, Aldrich) (MUA), and 16-mercaptohexadecanoic acid (90%, Aldrich) (MHDA) were all used as received. All other chemicals were p.a. grade (Merck). Buffer solutions (pH 3.5−6) were prepared by combining sodium acetate with concentrated solutions of hydrochloric acid and Millipore MilliQ water (resistivity > 18.2 MΩ cm). The concentration of the buffer solutions ranged from 1 to 100 mM.

**Electrochemical Apparatus and Procedures.** An Autolab PGstat 20 potentiostat was used for cyclic voltammetry. A homemade three-electrode cell consisting of a gold working electrode, typically a wire with an attached bead, a platinum wire counter electrode, and a $Hg|Hg_2SO_4$ reference electrode, was employed. All potentials reported in this paper are relative to the standard hydrogen electrode (SHE). All solutions were deaerated by purging with argon for 15 min. For voltammograms at high scan rates (>5 V/s) that were used in the determination of electron-transfer rate constants, ohmic drop compensation was applied. Ligand binding experiments were performed in saturated solutions of carbon monoxide (purity 4.7) or nitric oxide (purity 2.5, Linde AG) by purging the solution for 10 min. Prior to entering the electrochemical cell, NO was bubbled through two washing flasks filled with a 3 M KOH solution, a procedure that was found to be important to remove $NO_2$.[36,37] The saturated solutions contained 2.1 mM NO or 1.1 mM CO at 20 °C.[38] All electrochemical experiments were performed at room temperature.

**Preparation of Gold Electrodes with COOH-Terminated Thiols.** Prior to use, the gold wire electrodes were flame-annealed and subsequently quenched in water. The electrodes were then immersed in a 1 mM solution of one of the COOH-terminated thiols for approximately 10 min. For 3-mercaptopropionic acid and 4-mercaptobutyric acid, these solutions were prepared by mixing the thiol with water. The other thiol solutions were prepared by mixing with ethanol. The gold electrodes were rinsed and subsequently immersed in the electrochemical cell. The protein concentration in the cell was 100 nM. Protein adsorption was enhanced by argon or CO bubbling and continued until the voltammetric peak reached a maximum, which was after approximately 5 min in a solution of pH 4.5.

**Determination of Protein Coverages.** Coverages were determined by subtraction of a natural cubic splines baseline[39] from the anodic or cathodic scan of the voltammogram and subsequent division by area of the gold electrode. For the baseline subtraction, the program "Utilities for Data Analysis" developed by Dr. Dirk Heering was employed.[40] The surface area of the gold electrodes was determined from an oxygen adsorption experiment in 0.5 M $H_2SO_4$.[41]

**UV−Vis Spectroscopy.** UV−vis spectra were obtained at room temperature on a Shimadzu Multispec-1501 using a quartz cuvette with a rubber septum. Cytochrome *c*′ was diluted from a concentrated stock solution to a concentration of 1 μM in 50 mM acetate, pH 4.5. The solution was saturated with CO by flushing with CO. Cytochrome *c*′ was reduced by the addition of ∼15 mM $Na_2S_2O_4$. Subsequently, the increase in absorption at 418 nm with time was measured.

## Results

**Adsorption of Cytochrome *c*′.** Figure 1 shows the adsorption of cytochrome *c*′ from a 100 nM solution on a gold electrode with a SAM of mercaptohexanoic acid at pH 4.5. The peak size gradually increases with the number of scans, with full coverage being reached after approximately 100 scans, corresponding to 2000 s. The shape of the peaks is typical of an adsorbed species and does not display any diffusion limitation, which is a strong indication that the peaks are only caused by adsorbed cytochrome *c*′. When argon is bubbled through solution to enhance convection, the maximum coverage is obtained within 15 scans, corresponding to 300 s.

Figure 2 shows baseline-subtracted voltammograms for cytochrome *c*′ and cytochrome *c* adsorbed at pH 4.5. Potentials

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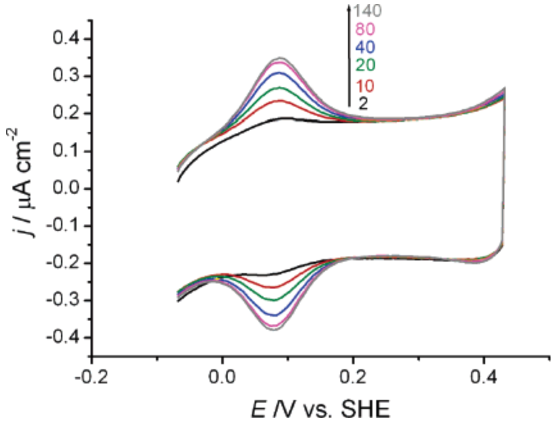


**Figure 1.** Cyclic voltammograms of a gold electrode with a SAM of mercaptohexanoic acid (MHA) after immersion in 100 nM *Allochromatium vinosum* cytochrome *c′* in a 10 mM acetate solution, pH 4.5. Scans 2, 10, 20, 40, 80, and 140 are displayed. Scan rate = 50 mV/s.

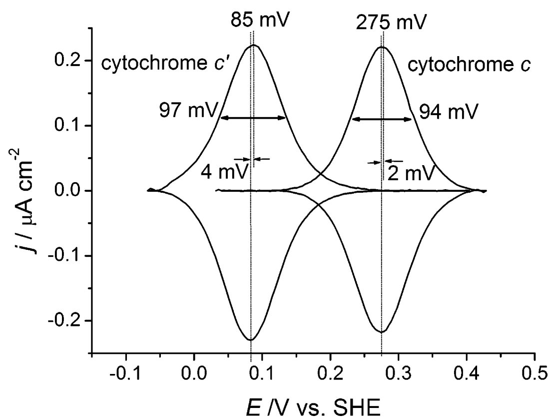


**Figure 2.** Baseline-subtracted voltammograms of a gold electrode coated with mercaptohexanoic acid (MHA) after immersion in 100 nM *Allochromatium vinosum* cytochrome *c′* or 200 nM horse heart cytochrome *c* in a 10 mM acetate solution, pH 4.5. Scan rate = 50 mV/s.

of 85 and 275 mV were determined for the $Fe^{III}/Fe^{II}$ transitions of cytochrome *c′* and cytochrome *c*, respectively. The 190 mV difference between both proteins reflects the different coordination of the heme, being five-coordinate mixed-spin for cytochrome *c′* and six-coordinate low-spin for cytochrome *c*.[42−44] A difference of 190 mV has also been observed between the midpoint potentials of the two proteins in solution, which are 60 mV for cytochrome *c′*[45,46] and 250 mV for cytochrome *c* at pH 4.5.[47] The difference of 25 mV between the midpoint potentials of the immobilized proteins as compared to the proteins in solution is probably related to the negative charge of the carboxyl thiol and the electric field created by the gold electrode.[25]

Half-height widths ($\delta$) are 97 mV for cytochrome *c′* and 94 mV for cytochrome *c*. Both $\delta$'s are close to the theoretical $\delta$ of 89 mV at 21 °C for Nernstian behavior[48] and are similar to $\delta$'s observed for proteins immobilized on edge plane pyrolitic graphite.[49] This implies that the immobilized protein molecules display almost ideal electron-transfer behavior, which is also reflected in the small peak separation between the anodic and the cathodic peaks, being 4 mV for cytochrome *c′* and 2 mV for cytochrome *c*.

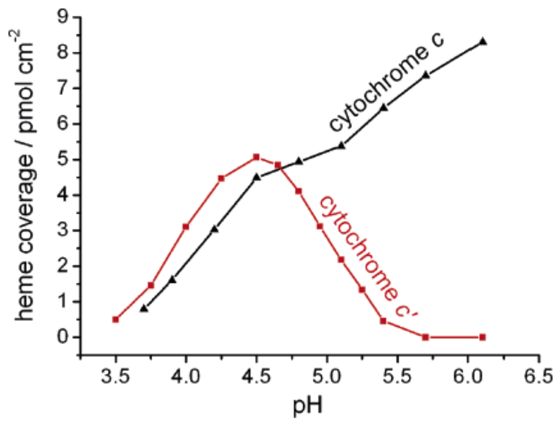


**Figure 3.** Heme coverages of cytochrome *c′* (■) and cytochrome *c* (▲) determined at different pHs on SAMs of mercaptohexanoic acid on gold. Coverages were determined from the charge under the voltammetric peaks of baseline-subtracted voltammograms divided by the surface area of the gold electrodes. These surface areas were determined from oxygen adsorption experiments.[41] The voltammograms were recorded in 10 mM acetate solutions containing 100 nM cytochrome *c′* or 200 nM cytochrome *c* at a scan rate of 50 mV/s. The actual protein coverage for cytochrome *c′* is one-half the heme coverage.

On the basis of the charge under the voltammetric peaks and the surface area of the gold electrodes, the heme coverage can be determined. Because cytochrome *c′* is a dimer, the actual protein coverage is one-half the heme coverage. It appeared from the voltammograms that there was a strong influence of pH on heme coverage (Supporting Information S1). Therefore, the maximum heme coverage for cytochrome *c′* and cytochrome *c* was determined as a function of pH. Figure 3 shows that there is a gradual increase in coverage up to pH 4.5 for both proteins. At pH's above 4.5, the coverage of cytochrome *c′* decreases and becomes zero at pH 5.5. The maximum heme coverage for cytochrome *c′* is 5 pmol $cm^{-2}$ at pH 4.5. The coverage of cytochrome *c* keeps increasing above pH 4.5 and does not reach a maximum. The value of 8.5 pmol $cm^{-2}$ at pH 6 is in line with previously reported values of 10−15 pmol $cm^{-2}$ at pH 7.[14,21,22,50]

Our results can be rationalized, taking into account the fact that high coverages are only obtained if the thiol is sufficiently negatively charged and the protein is sufficiently positively charged. Cytochrome *c* has a pI of 10.0 and is therefore sufficiently positively charged over the whole pH range. Cytochrome *c′* has a pI of 4.9 and hence is only positively charged below pH 4.9, which explains the sharp decrease in coverage above pH 4.5. The increase in coverage observed in the pH 3.5−4.5 range for both proteins can be related to an increase in the negative charge on the carboxylic acid-terminated SAM. Carboxylic acids have a $pK_a$ around 4.8,[38] but higher $pK_a$'s have been reported for SAMs of carboxylic acid-terminated thiols.[18,51−60]

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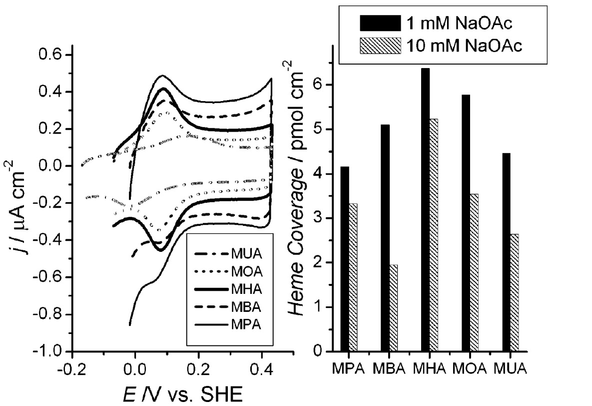


**Figure 4.** Cyclic voltammograms and heme coverages for a gold electrode coated with a SAM of 3-mercaptopropionic acid (MPA), 4-mercaptobutyric acid (MBA), 6-mercaptohexanoic acid (MHA), 8-mercaptooctanoic acid (MOA), or 11-mercaptoundecanoic acid (MUA) in 100 nM *Allochromatium vinosum* cytochrome *c*′ in 10 mM acetate solution of pH 4.5. Scan rate = 50 mV/s. Coverages obtained in buffer solutions of 1 mM acetate solution of pH 4.5 are also listed.

The electrostatic binding of cytochrome *c*′ is also reflected in the fact that the coverage depends on both the length of the carboxylic acid-terminated thiol and the ionic strength of the solution. Figure 4 shows different coverages on thiols of different length, which may be related to a difference in p$K_a$ values between the SAMs.[51] Significantly higher coverages for all SAMs are obtained in a 1 mM buffer instead of a 10 mM buffer, which is also indicative of electrostatic binding. The binding of cytochrome *c*′ is weaker than that of cytochrome *c*, because the coverage of electrostatically bound cytochrome *c* does not decrease until the buffer concentration is raised over 25 mM (Supporting Information S2).[21,50,61]

**Electron Transfer to Immobilized Cytochrome *c*′.** Having established proper conditions for electrostatic immobilization of cytochrome *c*′ on SAMs, we next studied the rate of electron transfer as a function of thiol length. Studies on electrostatically immobilized cytochrome *c* previously showed a distinct influence of the length of the carboxylic acid-terminated thiol on the electron-transfer rate (Figure 6),[22,23] displaying rates limited by electron tunneling for long thiols and rates limited by protein reorganization for short thiols. Electron-transfer rate constants on the different thiols were determined employing the method described by Laviron.[62] This involves measuring the cathodic and anodic peak potentials of voltammograms at different scan rates (Supporting Information S3) and subsequent plotting of these potentials in so-called Trumpet plots (Figure 5).[63] From these plots, rate constants at midpoint potentials of 140, 140, 36, 3.9, and 0.18 $s^{-1}$ were determined for SAMs of, respectively, 3-mercaptopropionic acid (MPA), 4-mercaptobutyric acid (MBA), 6-mercaptohexanoic acid (MHA), 8-mercaptooctanoic acid (MOA), and 11-mercapundeanoic acid (MUA).[62,63] The determined rate constants are plotted in Figure 6 as a function of chain length. For comparison, electron-transfer rate constants were also determined for horse heart cytochrome *c* under the same

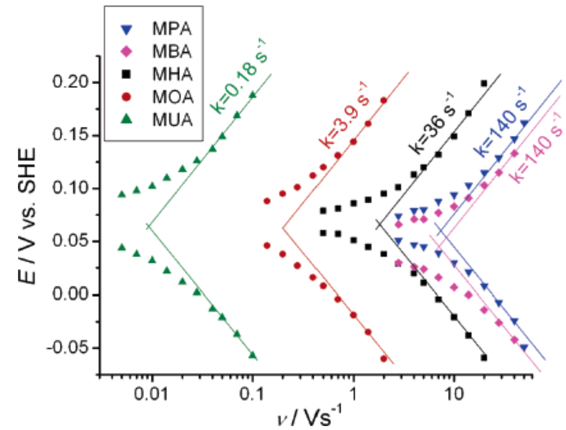


**Figure 5.** Trumpet plots for cytochrome *c*′ immobilized on gold with a SAM of 3-mercaptopropionic acid (MPA), 4-mercaptobutyric acid (MBA), 6-mercaptohexanoic acid (MHA), 8-mercaptooctanoic acid (MOA), or 11-mercaptoundecanoic acid (MUA). Cathodic and anodic peak potentials were determined from voltammograms that were recorded in 10 mM acetate, pH 4.8, containing 100 nM cytochrome *c*′ at scan rates ranging from 5 mV $s^{-1}$ to 40 V $s^{-1}$. Peak potentials were corrected for ohmic drop at high scan rates. The corresponding electron-transfer rate constants are also reported.

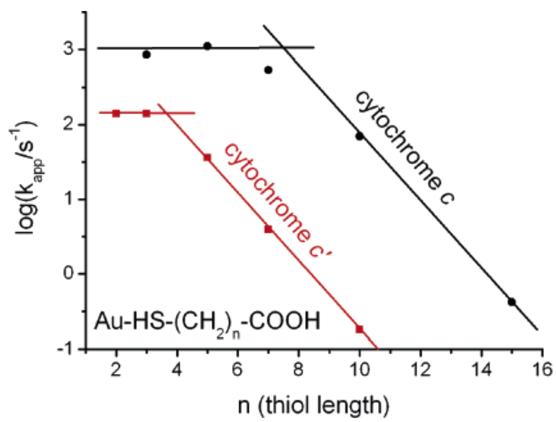


**Figure 6.** Electron-transfer rate constants for cytochrome *c*′ and cytochrome *c* as a function of the number of methylene groups in the mercaptocarboxyl thiols ($HS(CH_2)_nCOOH$). The electron-transfer rates were determined from Trumpet plots (Figure 5). The voltammograms for these Trumpet plots were recorded in 10 mM acetate, pH 4.8, containing 100 nM cytochrome *c*′ or 200 nM cytochrome *c* at scan rates ranging from 5 mV $s^{-1}$ to 300 V $s^{-1}$. For cytochrome *c* on SAMs of MBA, MHA, and MOA, a 50 mM acetate solution was used to avoid large ohmic drops. Increasing ionic strength does not affect rate constants, as was evidenced by values of 70 and 75 $s^{-1}$ for cytochrome *c* on MUA in 10 and 50 mM acetate, respectively.

conditions. The determined values of 845, 1098, 536, 69.5, and 0.42 $s^{-1}$ for, respectively, MBA, MHA, MOA, MUA, and 16-mercaptohexadecanoic acid (MHDA) are in close correspondence with values previously reported for cytochrome *c* at pH 7.[22]

Figure 6 shows that cytochrome *c*′ and cytochrome *c* exhibit a similar kind of electron-transfer behavior. For long thiols, the electron-transfer constant decreases with increasing length of the thiol, corresponding to long-range electron tunneling.[64] The slope of this decrease is the same for both cytochrome *c* and cytochrome *c*′ and corresponds to an exponential decay coefficient $\beta$ of 1.06 per methylene unit, in line with electron-transfer rates through alkanethiols reported for other systems.[65–67] For short

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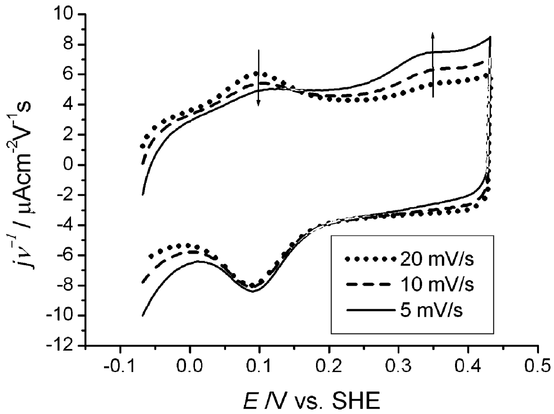


**Figure 7.** Cyclic voltammograms at 20 mV/s (−), 10 mV/s (- - -), and 5 mV/s (• • • •) of a gold electrode with a SAM of 6-mercaptohexanoic acid in a saturated CO solution (∼1.1 mM) with 100 nM cytochrome *c*′ in 1 mM acetate, pH 4.5. For comparison, voltammograms are divided by scan rate.

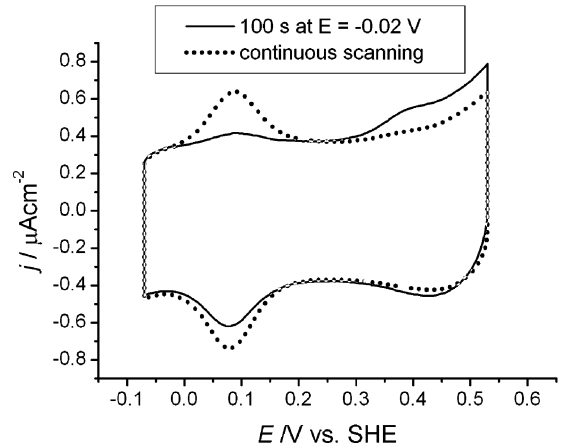


**Figure 8.** Cyclic voltammograms of a gold electrode with a SAM of 6-mercaptohexanoic acid in a saturated CO solution with 100 nM cytochrome *c*′ in 10 mM acetate, pH 4.5. The dotted line (• • • •) is obtained after continuous scanning at 100 mV/s. The solid line (−) is obtained in the first scan (100 mV/s) after incubation at $E = -0.02$ V vs SHE for 100 s.

thiols, the electron-transfer rate constants become independent of chain length for both proteins. This has been ascribed to a rate-limiting conformational change or proton transfer.[68]

However, there are some distinct differences between immobilized cytochrome *c*′ and immobilized cytochrome *c*. For short thiols, the electron-transfer rate constant of immobilized cytochrome *c*′ (∼150 s$^{-1}$) is a factor 7 slower than the rate constant of immobilized cytochrome *c* (∼1000 s$^{-1}$). This difference implies that the conformational change or proton transfer accompanying the electron transfer is slower for cytochrome *c*′ than for cytochrome *c*, which is not surprising because cytochrome *c* is an electron-transfer protein optimized to efficiently undergo redox changes, whereas the function of cytochrome *c*′ is still unclear.[42,43] For long thiols, the rate constants for immobilized cytochrome *c*′ are even 500-fold slower than those for immobilized cytochrome *c*. This suggests that the heme group of immobilized cytochrome *c*′ is further away from the electrode than the heme group of immobilized cytochrome *c*.

**Ligand Binding to Cytochrome *c*′.** The efficient electron transfer between the electrode and the heme groups in cytochrome *c*′ also allowed us to study the effects of ligand binding on cytochrome *c*′. CO, NO, and CN$^-$ have all been reported to induce protein monomerization, which is caused by a conformational change in one of the α-helices present at the dimer interface that occurs upon displacement of Tyr16 by ligand binding.[29] We focused on CO because this ligand is easily handled and redox inactive. CO only binds to the protein in its Fe$^{II}$ state and was previously reported to bind relatively slowly.[44] Indeed, voltammograms recorded at high scan rates in a saturated CO solution do not show an apparent influence of CO, which is consistent with slow CO binding. However, when lowering the scan rate, we observed a decrease in the intensity of the original anodic peak and the formation of a new anodic peak at potentials about 250 mV more positive (Figure 7). This new peak can be assigned to the oxidation of Fe$^{II}$−CO cytochrome *c*′. Because Fe$^{II}$−CO cytochrome *c*′ has a six-coordinated heme, it has a much more positive potential. The reactions that occur are described in eqs 1−4. The fact that the size of the new anodic peak strongly depends on the scan rate implies that binding of CO to the Fe$^{II}$ state of cytochrome *c*′ is a relatively slow process. Because no new cathodic peak is observed at high potentials for any scan rate, we can also deduce that release of CO after oxidation of the Fe$^{II}$−CO cytochrome *c*′ is fast.

$$Fe^{III} + e^- \rightleftarrows Fe^{II} \qquad (1)$$

$$Fe^{II} + CO \rightarrow Fe^{II}{-}CO \qquad (2)$$

$$Fe^{II}{-}CO \rightarrow Fe^{III}{-}CO + e^- \qquad (3)$$

$$Fe^{III}{-}CO \rightarrow Fe^{III} + CO \text{ (fast)} \qquad (4)$$

Binding of CO does not only result in a shift of the anodic peak, but also results in significant conformational changes of the protein on the surface. This can be derived from Figure 8, where a voltammetric scan at 100 mV/s is plotted after the electrode is held at $E = -0.02$ V vs SHE for 100 s. At this potential, the immobilized cytochrome *c*′ is in its Fe$^{II}$ state, which means that it is slowly binding CO. Accordingly, in the voltammetric scan following the incubation period, the original anodic peak has largely disappeared and the new anodic peak corresponding to oxidation of Fe$^{II}$−CO is observed at higher potentials. This peak is found at approximately 0.38 V, which given the Nernstian behavior of the protein would imply that almost all CO-bound protein molecules should be oxidized by the time the voltammetric scan reaches its anodic limit of 0.53 V. However, there seems to be some tailing of the anodic peak, which could indicate limited electronic coupling between the protein and the electrode and hence suggests that part of the CO-bound cytochrome *c*′ has reoriented or desorbed. This is confirmed by the fact that the charge under the subsequent cathodic peak has significantly decreased, which implies that there is a decrease in coverage of electroactive protein. Experiments in the absence of CO did not show any loss in protein coverage when the electrode was held at $E = -0.02$ V vs SHE. A plausible explanation for the decrease in protein coverage is that the interactions between cytochrome *c*′ and the SAM are weaker in its monomeric form than in its dimeric form, resulting in a lower protein coverage. Another explanation is that the monomerization induced by CO binding causes the protein to reorient on the surface impeding the electron transfer between the electrode and the protein. This is also suggested by the fact that the position of the new anodic peak shifts positively with increasing scan rate, which indicates that electron transfer is relatively slow (Supporting Information S4). This makes it difficult to investigate the new anodic peak of the

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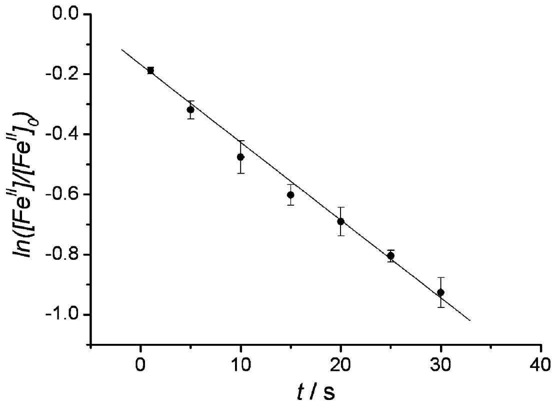


**Figure 9.** Plot of the natural logarithm of the fraction of $Fe^{II}$ that has not reacted with CO versus the time at which the potential has been kept at $E = -20$ mV vs SHE. Fractions were determined by dividing the low potential anodic peak area after incubation by the anodic peak area before incubation. Voltammograms were recorded at 1 V/s. The experiments were performed on cytochrome *c′* immobilized on a self-assembled monolayer of mercaptohexanoic acid in a 10 mM acetate solution, pH 4.5. All points were measured at least three times, and standard deviations are indicated by bars. $T = 22$ °C.

CO-bound cytochrome *c′* at high scan rates, because it shifts outside our potential window. Both explanations are consistent with the CO-induced monomerization observed in solution and suggest that protein monomerization is not impeded by the fact that the protein is immobilized. Denaturation of the protein does not occur, as is reflected in the fact that both the cathodic and the anodic peaks are recovered after continued scanning.

To determine the rate of CO binding to the adsorbed cytochrome *c′*, we measured the binding of CO as a function of time. Analogous to Figure 8, the potential of the electrode was held at −0.02 V vs SHE for a certain time, and subsequently a scan at 1 V/s was recorded. A high scan rate was used to avoid readsorption of cytochrome *c′* from solution during the scan. The voltammograms are displayed in Supporting Information S5. Direct determination of CO binding employing the area under the new anodic peak is not possible, because not all CO-bound cytochrome *c′* is oxidized within our potential window at this high scan rate. Therefore, we determined the amount of adsorbed cytochrome *c′* that had not reacted with CO by integration of the low potential anodic peak. The area under this peak was divided by the area under the anodic peak before incubation at $E = -0.02$ V, resulting in the fraction of cytochrome *c′* that has not reacted with CO. In Figure 9, the natural logarithm of this fraction is plotted as a function of the incubation time. A linear relationship is observed, which means that binding of CO to cytochrome *c′* is a first-order process. Such a process can be described by eq 5. Given the saturation concentration for CO of 1.1 mM, a $k$ of $24 \pm 1$ $M^{-1}$ $s^{-1}$ can be deduced.

$$\ln\left(\frac{[Fe^{II}]}{[Fe^{II}]_0}\right) = -k[CO]t \tag{5}$$

To determine whether immobilization of the protein affects its CO binding rate, we also measured the CO binding to cytochrome *c′* in solution under identical conditions. For this, the absorption maximum at 418 nm typical of CO-bound cytochrome *c′* was monitored by UV/vis spectroscopy.[35] Figure 10 shows a gradual increase in the absorbance at this wavelength after reduction of ferric to ferrous cytochrome *c′* by dithionite in a CO saturated solution. The data can be fitted with a first-order exponential decay, which implies that the reaction is first

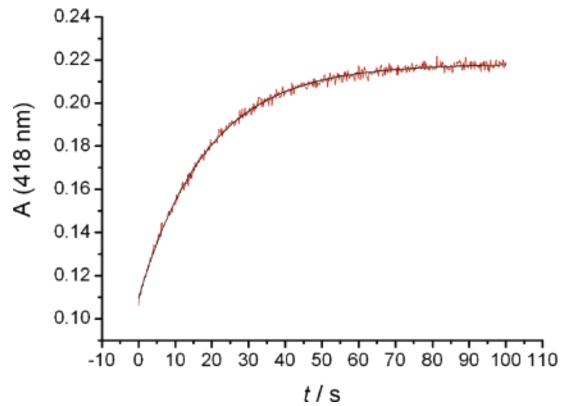


**Figure 10.** CO binding to cytochrome *c′* measured by UV/vis spectroscopy. Absorption at 418 nm (typical of CO-bound cytochrome *c′*) is followed in time after ferric cytochrome *c′* is reduced to ferrous cytochrome *c′* by dithionite in a CO saturated solution. The solution contained 1 $\mu$M cytochrome *c′* in 50 mM acetate solution, pH 4.5. $T = 22$ °C. The data were fitted with a first-order exponential decay (black line).

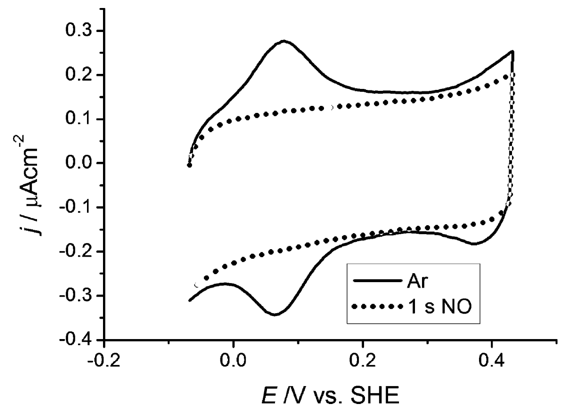


**Figure 11.** Cyclic voltammograms of cytochrome *c′* immobilized on a SAM of mercaptohexanoic acid before (−) and after 1 s of NO bubbling (• • • •) through an 10 mM acetate solution, pH 4.8, containing 100 nM cytochrome *c′*. Scan rate = 50 mV/s.

order in cytochrome *c′* with a $k$ of $48 \pm 1$ $M^{-1}$ $s^{-1}$. The measured rate is lower than the previously reported value of 140 $M^{-1}$ $s^{-1}$,[44] which is probably due to the difference in pH between both measurements (pH 7.4 vs pH 4.5). The fact that the CO binding rate in solution is of the same order as for immobilized cytochrome *c′* suggests that that the protein retains near-native behavior on the surface.

The NO binding properties of immobilized cytochrome *c′* are distinctly different from the CO binding properties. Bubbling NO through solution for just 1 s resulted in an immediate disappearance of the anodic and cathodic peaks (Figure 11). We could not detect a new anodic peak below 400 mV, but were able to recover a small fraction of the original protein peaks if we cycled at high scan rates to potentials as high as 800 mV vs SHE (Supporting Information S6). This is in line with the behavior of iron porphyrins, which can also only be oxidized at high potentials.[69,70] Because of anodic oxide formation on the electrode, we could not detect the location of a new anodic peak. Also, the NO binding rate is too fast to determine it voltammetrically, so we can only say that NO binding is much faster than CO binding. This is consistent with data reported for *Alcaligenes xylosoxidans* cytochrome *c′* in solution, which is the only cytochrome *c′* for

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which NO binding rates have been reported. For this cytochrome *c*′, NO and CO binding rates were determined to be 4400 and 92 $M^{-1}$ $s^{-1}$, respectively.[44,71]

## Discussion and Conclusions

We have successfully immobilized *Allochromatium vinosum* cytochrome *c*′ on carboxylic acid-terminated SAMs. The immobilization does not seem to induce large conformational changes or denaturation of the protein. We base this on the facts that (i) the potential for the $Fe^{III}/Fe^{II}$ heme transition is in reasonable accordance with values reported for the protein in solution, (ii) the coverage is similar to the coverage of immobilized cytochrome *c* and is typical of a protein, (iii) the voltammetric peaks display almost ideal Nernstian behavior, which is typical of a protein, but uncommon for, for example, adsorbed porphyrins,[72,73] (iv) the narrow pH range in which successful adsorption can be achieved is in accordance with the pI of the protein, (v) the observed electron-transfer behavior is typical of an immobilized protein, and (vi) the unique ligand binding properties observed for cytochrome *c*′ in solution (slow CO binding, fast NO binding) are also observed for immobilized cytochrome *c*′.

Our results on immobilized cytochrome *c*′ from *Allochromatium vinosum* distinctly differ from previous immobilization studies of cytochromes *c*′ from different organisms.[74−76] In these studies, peak separations of over 50 mV[74] and much lower electron-transfer rate constants were reported.[76] Additionally, protein adsorption rates were more than 10 000-fold slower than in our work.[76] Taking into account that that no dependence of ionic strength on protein coverage was observed,[76] it seems that the binding of cytochrome *c*′ in these studies was not electrostatic. This is also suggested by the fact that the measurements were reported at pH 7 (where the protein is negatively charged) on a negatively charged SAM. It is likely that the way cytochrome *c*′ was bound in these studies induced conformational changes to the proteins structure, which impedes the comparison with our results. Moreover, no electron transfer on thiols of different length and ligand binding kinetics were reported in these studies.

On the basis of our results and the structure of the protein, we can make a rough estimate of the way in which cytochrome *c*′ adsorbs on the carboxyl thiol layer. Binding of cytochrome *c*′ to the negatively charged SAMs occurs via positively charged residues (arginine and lysine) on the surface of the protein. Given the high heme coverage observed with cytochrome *c*′, we can assume that both hemes in the protein are in electronic contact with the electrode. It is unlikely that direct electron transfer occurs between the two hemes in cytochrome *c*′ due to the fact that the edge-to-edge distance between both hemes is 19 Å (the iron-to-iron distance is 23 Å), which is well above the maximum of 14 Å reported for feasible biological electron transfer.[77] Because we observe a single electron-transfer rate, we can conclude that both hemes are probably at an equal distance from the electrode. This leaves only two possible orientations by which the protein

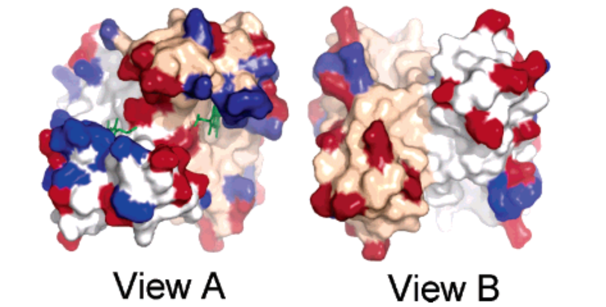


**Figure 12.** Charge distribution on cytochrome *c*′ at pH 5.0, observed from opposite sides of the protein. Positive groups (lysine and arginine) are depicted in blue, whereas negative groups (aspartic acid and glutamic acid) are depicted in red. The heme groups are depicted in green. Different colors are used for the two monomers forming the dimeric protein. PDB code: 1BBH.

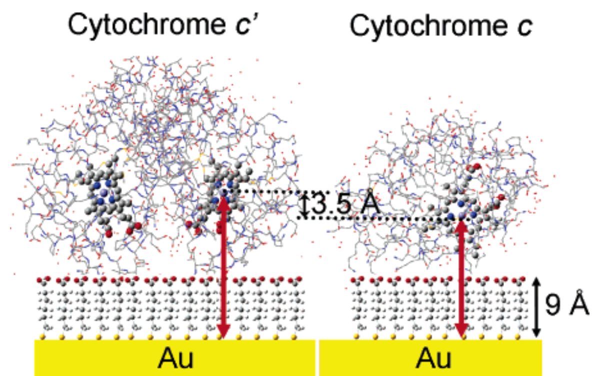


**Figure 13.** Schematic representation of *Allochromatium vinosum* cytochrome *c*′ (left) and horse heart cytochrome *c* (right) immobilized on a SAM of mercaptohexanoic acid. The heme groups in both proteins have been accentuated. The representation is approximately to scale. PDB code: 1BBH.

can bind to the SAM (Figure 12). Because the positively charged residues are mostly located on one particular side of the protein (view A), it is likely that the protein is bound to the electrode via this side. The hemes are located close to this positive surface, explaining why efficient electron transfer was observed between the electrode and the protein.

On the basis of the differences in electron-transfer rate constants between immobilized cytochrome *c*′ and cytochrome *c*, we can roughly estimate the difference in the heme−electrode distance between both systems. For this, we assume[78] that the difference in reorganization energy upon reduction of both proteins is reflected in the difference between the maximum electron-transfer rate constants observed at short thiols. Given the differences in electron-transfer rate constants of approximately 500 and 7 for long and short thiols, respectively, we can deduce that a 70-fold decrease is caused by the extra distance. Assuming that the exponential decay coefficient $\beta$ through both proteins is approximately 1.2 $Å^{-1}$,[79] we can estimate that this distance is about 3.5 ± 1 Å.[64] This agrees with the distance predicted from an analysis of the most likely orientation of both cytochrome *c*′ and cytochrome *c* immobilized on an electrode (Figure 13).

An important reason to study cytochrome *c*′ was to probe whether ligand-induced conformational changes are impeded when a protein is immobilized. In the case of cytochrome *c*′, these conformational changes can be induced by CO binding, which results in the monomerization of the protein. In

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voltammetry, we observed the formation of a new anodic peak at high potentials reflecting the oxidation of a newly formed $Fe^{II}-CO$ adduct. Additionally, we observed a decrease in the cathodic peak indicating the loss of electroactive protein. This suggests that the protein indeed undergoes monomerization, which influences the binding of the protein to the electrode. The protein either reorients on the surface with a possible temporal loss of electron transfer or desorbs. Although the difference in CO binding kinetics is relatively small, CO binding is slower for surface immobilized cytochrome *c*′ by a factor 2. This could be due to either the electrostatic interactions between the electrode and the protein or the steric hindrance on the surface hindering monomerization.

In conclusion, we have shown that cytochrome *c*′ immobilized on carboxylic acid-terminated SAMs retains a near-native conformation, exhibits fast electron transfer to the electrode, and probably undergoes the same conformational changes upon ligand binding as in solution. Our results suggest that carboxylic acid-terminated SAMs are suitable for the study of immobilized proteins in general, because the electrostatic interactions are sufficient to confine the protein to the surface, but do not significantly affect its properties. Successful immobilization requires that the protein is sufficiently positively charged and that the distance between the electrode and the proteins redox groups is not too long. Whether these requirements are met can be deduced from the pI of the protein and an analysis of the most likely conformation of the protein on the SAM.

**Acknowledgment.** This work was supported by the National Research School Combination Catalysis (NRSC-C).

**Supporting Information Available:** Voltammograms of immobilized cytochrome *c*′ at pH 3.8, 4.5, and 5.1, voltammograms of immobilized cytochrome *c* with increasing ionic strength, voltammograms of immobilized cytochrome *c*′ at increasing scan rates, voltammograms at increasing scan rates of CO-bound cytochrome *c*′, voltammograms employed for CO binding rate determination, and a voltammogram of immobilized cytochrome *c*′ up to 800 mV vs SHE in the presence of NO. This material is available free of charge via the Internet at http://pubs.acs.org.

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