Voltammetry of a 'protein on a rope'

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Abstract **A periplasmic electron-transfer protein, cytochrome c^{m}_{555} from *Aquifex aeolicus* contains a 62-residue N-terminal extension by which it is anchored to the membrane – most probably via a thioester bond to its N-terminal cysteine. This linker can act as a 'rope' to tether the protein close to its reaction partners. Mimicking this principle, a recombinant cytochrome c^{m}_{555}, expressed in *Escherichia coli*, has been attached covalently to a gold electrode modified with 6-mercaptohexan-1-ol. The 'tethered' cytochrome c^{m}_{555} displays remarkably fast electron-transfer kinetics, with an electrochemical exchange rate constant k_0 of 1.4×10^4 s^{-1}. The results show that fast electron transfer is associated with *weak* interactions: importantly, the tethered cytochrome can explore many different orientations without escaping into solution.**

Key words: Cytochrome; Voltammetry; Electron transfer; *Aquifex aeolicus*

1. Introduction

Many protein electron-transfer (ET) reactions depend on large interdomain motions, with well-established examples including the Rieske subunit of *bc* complexes [1,2] and flavocytochrome b_2 [3]. The importance of this type of control over biological ET rates is reinforced by increasing evidence that many small ET proteins, instead of being free to diffuse, are tethered permanently to a membrane or membrane-bound complex by a flexible linker. Examples include auracyanin B from *Chloroflexus aurantiacus* [4] and several membrane-attached monoheme cytochromes *b* [5,6], and *c* [7–10]. Kinetic evidence that ET activity is regulated by the restricted mobility of proteins has been obtained for cytochrome c_y from *Rhodobacter capsulatus* [7] and cytochrome c_z of green sulphur bacteria [9], while EPR results on partially ordered membranes have shown that cytochrome $b_{558/565}$ from *Sulfolobus solfataricus* [6] and cytochrome c^{m}_{555} (c555m) from *Aquifex aeolicus* [11] can adopt several or possibly even a continuum of orientations with respect to the membrane. With their redox partners, they can constitute 'supercomplexes', as proposed for membrane-attached cytochromes in *Bradyrizobia* [8] and Gram-positive bacteria [10]. The cytochromes c_{552} from *Paracoccus denitrificans* and c_{551} from *Bacillus* PS3 are integrated into a supercomplex additionally containing cytochrome *bc* complex and cytochrome oxidase [10,12]. In the case of *R. capsulatus* the membrane-tethered cytochrome plays a role in electron transfer from the cytochrome *bc* complex to the photosynthetic reaction center, in competition with a soluble cytochrome that is also expressed in the organism [7,13]. While these soluble and tethered cytochromes have competing functions in the cytochrome bc_1/aa_3-oxidase chain [7,14] they may have different specific functions in the nitrous and nitric oxide reductase activities [15,16].

The hyperthermophile *A. aeolicus* expresses, in addition to the membrane-tethered c555m, a soluble periplasmic cytochrome c^{s}_{555} (c555s) [11]. These two cytochromes are highly homologous in the heme binding domain, but c555m has a 62 amino acid N-terminal extension. A sequence comparison is shown in Fig. 1. The first 17 residues in each case constitute the signal peptide that directs translocation across the cytoplasmic membrane, and which is then cleaved to release the mature protein. The following 62 amino acid sequence present in c555m corresponds to the linker, which provides attachment to the membrane via a thioester bond between phospholipid and the terminal cysteine. The predominant residues in the linker are glutamate, glutamine and lysine, so that it is more hydrophilic than those in auracyanin B and cytochrome c_y, which contain, respectively, 35 and 42 amino acids, mainly alanine and proline [4,13]. So far, no direct evidence concerning the functional roles or kinetic competence of c555m and c555s in *A. aeolicus* has been obtained. Their reduction potentials suggest that one or both might be involved in electron transfer from the cytochrome *bc* complex towards terminal oxidases [11].

Protein film voltammetry (PFV) provides detailed and integrated kinetic and thermodynamic information on the ET properties of redox proteins [17,18]. Here, the protein sample is adsorbed on an electrode, up to monolayer coverage, in such a way that electron exchange with the active sites is fast. The electrode is effectively a redox partner, delivering or removing electrons, but with the added advantage of being able to vary and control the potential (driving force) and measure the corresponding rates. The kinetic parameters of the redox reaction are readily extracted. In achieving this aim, there is considerable interest in electrodes (particularly Au) that are modified with a self-assembled monolayer (SAM) of thiol-containing adsorbates. The terminal functionalities on these adsorbates are usually designed to provide a surface that will give strong non-covalent interactions with the protein molecules and orient them for fast electron transfer [19–21].

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                                  ↓
c555m   MKLRYVFLSALAKGIFA[17]CEQPQKKEETKKEEPKKEAVAQ[39]
c555s   MKKLLLAGLAVAGLTFA----------------------

c555m   KQEAKKEEQKPQEQPKQEQKQEVKQEEQKQVAQAGGAGNP[79]
c555s   ----------------------------------------

c555m   EKGKAIFQQKGCGFCHQPAVDTVGPSLKKIAQAYAGKEDQLVK[122]
c555s   ADGKAIFQQKGCGSCHQANVDTVGPSLKKIAQAYAGKEDQLIK

c555m   FLKGEAPAIVDPAKEAIMKPQLMQLKGLSEQELRDLAAFIMSHK[166]
c555s   FLKGEAPAIVDPAKEAIMKPQLTMLKGLSDAELKALADFILSHK
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Fig. 1. Sequence comparison between cytochrome c^{m}_{555} (c555m) and cytochrome c^{s}_{555} (c555s) from *A. aeolicus*. Bold: residues that differ between the two sequences, with the cysteine highlighted by an arrow. Underlined: heme binding site and sixth ligand of the heme; italics: charged residues. The shaded regions indicate stretches of the linker sequence predicted to have helical structure (Pôle Bio-Informatique Lyonnais for Network Protein Sequence Analysis).

We have now exploited the presence of the cysteine on the linker arm to attach c555m *covalently* to an Au electrode, the idea being that the protein is now 'tethered' in a flexible fashion, resembling the situation in the bacterial membrane. As we describe here, our voltammetric studies of 'a protein on a rope' provide a novel way to examine the influence of this constraint and enhance our understanding of its physiological role.

2. Materials and methods

Cytochrome c555s was purified from *A. aeolicus* as described in [11]. Cytochrome c555m was expressed in *Escherichia coli* using the expression system developed for c555s [22] and purified from the soluble fraction by chromatography. N-terminal sequencing showed the presence of the entire protein sequence, including the precursor. The fact that the protein was present in the soluble fraction indicated that no lipid is attached to the cysteine residue.

The electrochemical cell, incorporating a saturated calomel electrode (SCE) as reference, and procedures for fast scan cyclic voltammetry using the Autolab electrochemical workstation (EcoChemie, Utrecht, The Netherlands) were as described previously [23]. Compensation for IR drop was carried out using the positive feedback IR compensation function of the Autolab analyzer, set at values just below that at which current oscillations appear. Gold electrodes were cleaned as described elsewhere [24] and modification with a monolayer of 6-mercaptohexan-1-ol (Fluka) was carried out by incubating the electrode in a 1 mM solution of thiol in ethanol at room temperature for at least 4 h. The electrode was rinsed thoroughly, and placed in a solution of c555s (50 μM) for 1 h, or c555m (20 μM) for at least 6 h; then it was rinsed with buffer to remove all protein molecules that were not immobilized on the surface. PFV experiments were performed in MOPS or phosphate buffers, pH 6.9, at 20°C, and the ionic strength was adjusted by varying the concentrations of buffer and $NaClO_4$. All potentials were corrected to conform to the standard hydrogen electrode (SHE) scale, using $E(\mathrm{SHE}) = E(\mathrm{SCE}) + 243$ mV at room temperature [25]. Cyclic voltammograms were typically recorded from −0.067 to +0.543 V at different scan rates, and no significant differences were noted between voltammograms commenced at the reducing or oxidizing limits. Thiol electrodesorption voltammograms were obtained by placing the modified electrode into 0.1 M NaOH and sweeping the potential over ranges of approximately 0.2 to −1.2 V vs. SHE at 50 mV s^{-1} [21,26].

3. Results

Fig. 2 shows electrodesorption voltammograms (obtained in 0.1 M NaOH) for gold electrodes pre-incubated in the presence of 6-mercaptohexan-1-ol, or c555m, or one incubated first with 6-mercaptohexan-1-ol then c555m. These voltammograms reveal the presence of thiol groups that are covalently attached to the electrode: their reductive desorption (release of thiolate is induced by the reduction of surface Au(1) to Au(0) atoms) gives rise to voltammetric waves, the potentials of which represent different types of thiol and the stability of the self-assembled monolayers they form [21,26]. The gold electrode incubated with 6-mercaptohexan-1-ol gives a single wave at −0.96 V, while one incubated with a solution of c555m shows a wave at −0.92 V. The third electrode, pre-incubated first with 6-mercaptohexan-1-ol then c555m shows two waves. While these are shifted compared to the voltammograms obtained with pure adsorbates, the facts that two separate waves are obtained, and that no signal is detected when c555s is used in place of c555m, indicate that c555m is attached to the gold electrode by a thiol group. Shifts in potential when mixtures are studied are commonly observed in this type of experiment [21]. As described next, the presence of a stable bond between the gold surface and c555m, but not c555s, is confirmed by the protein film voltammetry.

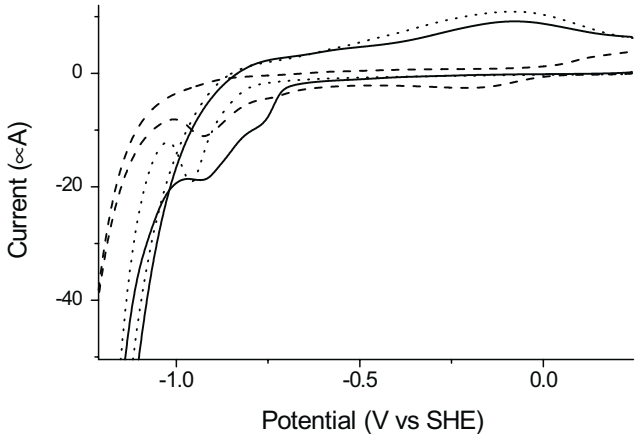

Fig. 2. Desorption voltammograms of gold electrodes modified with 6-mercaptohexan-1-ol (dotted line), c555m only (dashed line), 6-mercaptohexan-1-ol and c555m (bold line).

Fig. 3 shows baseline-corrected cyclic voltammograms for films of c555s and c555m formed on an Au electrode modified with a SAM of 6-mercaptohexan-1-ol. No response was obtained for c555m attached to a bare gold electrode, even

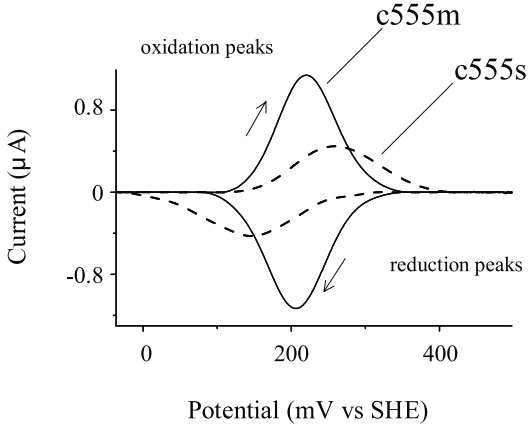

Fig. 3. Cyclic voltammograms (baseline-corrected) of c555m (bold lines) and c555s (dotted lines) on a 6-mercaptohexan-1-ol-modified electrode at an ionic strength of 0.5 M and a scan rate of 7.8 V s^{-1}. Temperature 20°C. The arrows indicate the direction of cycling.

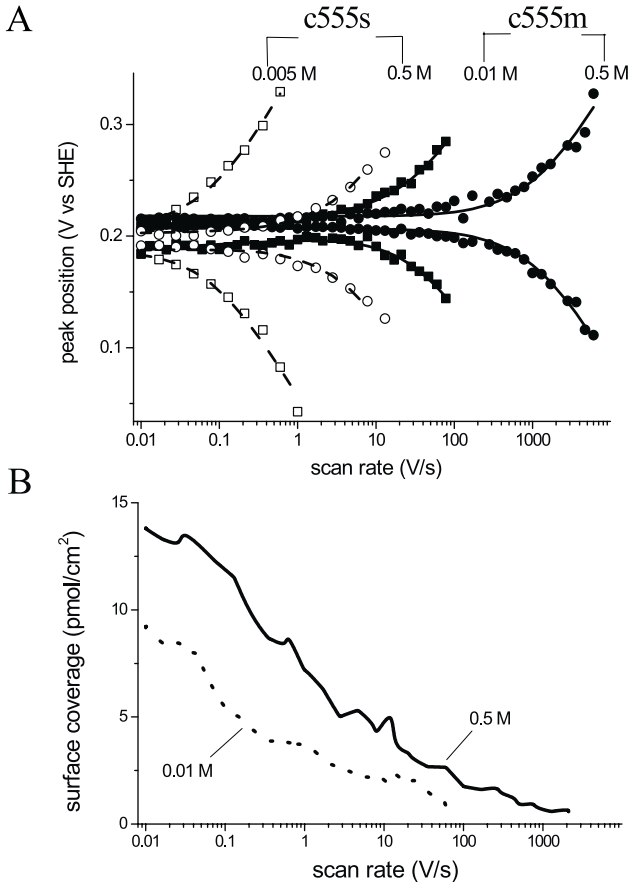

Fig. 4. A: 'Trumpet plots' of the voltammetric data obtained for c555s (dashed lines, open symbols) and c555m (bold line, closed symbols) at different ionic strengths. Temperature 20°C. B: The active surface coverage (relating to the number of molecules contributing to the peaks) of c555m on a 6-mercaptohexan-1-ol-modified gold electrode measured at different scan rates and ionic strengths of 0.01 M (dotted line) and 0.5 M (bold line). Temperature 20°C.

though the presence of the covalently attached protein was evident from the desorption voltammogram (see Fig. 2). For c555s the stability of the protein film decreased rapidly with increasing ionic strength (I), whereas the films of c555m were stable even at $I = 2$ M. These voltammograms were measured at an intermediate scan rate (7.8 V s^{-1}) and it is evident, from the much smaller separation of oxidation and reduction peaks, that electron transfer is much faster for c555m.

Fig. 4A shows the resulting 'trumpet plots', i.e. variations of the peak positions with (log) scan rate [18,23] and Fig. 4B the corresponding variations in electroactive coverage of c555m as a function of scan rate [27]. The electroactive coverage, obtained by determining the area under the peak and computing the charge passed from the scan rate used, reflects the number of molecules able to transfer electrons sufficiently rapidly to make a contribution within the peak area. Each data set was collected with the same electrode in the directions of both increasing and decreasing scan rates. From respective onsets of peak divergence in Fig. 4A it is clear that ET for c555m is about two orders of magnitude faster than for c555s. Fits to the Butler–Volmer equation yield values for the formal rate constant k_0, which is the rate of electron exchange between electrode and active site when the driving force is zero (i.e. at the formal reduction potential) [23,24,27,28]. Values for c555s were: 1 s^{-1} and 30 ± 2 s^{-1} at $I = 0.005$ M and 0.5 M, respectively, while for c555m, rate constants of 300 ± 200 s^{-1} and $14\,000 \pm 3000$ s^{-1} were obtained at $I = 0.01$ M and 0.5 M. The plot in Fig. 4B shows clearly that the number of molecules of c555m being addressed at scan rates sufficiently fast to define the ET rate (1–2 pmol cm^{-2} at 1000 V s^{-1}) is much lower than the number observed at slow scan rates (15 pmol cm^{-2}). Half-height peak widths lay between 85 and 99 mV (< 1 V s^{-1}) and 83 and 95 mV (< 10 V s^{-1}) for c555s and c555m respectively, indicative of a high degree of chemical homogeneity among the molecules able to exchange electrons with the electrode [27]. At higher scan rates, widths became difficult to measure accurately as the electroactive coverage diminished, although even at 1000 V s^{-1}, peaks were still clearly visible above the capacitance background.

4. Discussion

The electrodesorption peak observed for c555m but not for c555s suggests strongly that this protein is linked to the electrode via a thiol group, the only reasonable option for which the cysteine is located in the linker region. Further support for a stable covalent linkage stems from the ionic strength dependence of the protein film voltammetry: thus, while c555s dissociates as I is raised, c555m remains strongly bound at $I = 0.5$ M and signals persist for hours after the preparation of the film (signals from c555s are lost within minutes under these conditions). There is therefore compelling evidence that c555m is tethered to the electrode, via the thiol group of cysteine. Significantly, no electron transfer is detected unless the electrode is also modified with the thiol SAM.

With this assumption, we now compare the kinetics for c555m and c555s which are very different, noting that the k_0 value of 14 000 s^{-1} obtained for c555m is the fastest value yet reported for a protein at an electrode. There are several related points to consider.

(A) The sequence of c555s is very similar to that of the heme binding (globular) domain of c555m: 74 out of 87 amino acid residues are identical, with the globular part of c555m having just one additional positively charged amino acid compared to c555s. We therefore expect that the three-dimensional (3-D) structures and surface charges of both cytochromes are also similar and the differences observed can mainly be attributed to the presence of the linker sequence in c555m.

(B) Films of c555s are unstable, especially at high I, suggesting that polar attraction forces are important in binding the protein to the electrode.

(C) The ET rate constants for both c555s and c555m increase as I is raised.

(D) As the scan rate is increased, the fraction of c555m molecules able to exchange electrons rapidly within the diminishing time span (i.e. to contribute to the peak) drops dramatically.

These observations provide compelling evidence for a model in which the polar protein–electrode interactions that are strengthened at low I are associated with less favorable orientations for ET. The model embraces a dynamic description of the binding of a protein at a surface where it undergoes a large number of translational and rotational movements before dissociation occurs. Upon dissociation, a soluble protein escapes irreversibly, whereas a tethered protein will return frequently to the electrode surface. The tethered protein is

therefore able to explore a wider range of different orientations, even those that are only weakly supported by interactions between the protein and the electrode. Configurations having better ET coupling are thus visited more frequently. We propose that in our case, *fast* ET is associated with *weak* interactions between the cytochrome and the SAM. An additional possibility is that steric constraints due to the position of the linker prevent excursions of c555m into less active orientations. The importance of protein dynamics, as discussed in several papers [29–32] is established further from the fact that the number of c555m molecules detected on the electrode depends on scan rate. Clearly, within the timescale (approximately 100 μs) of experiments carried out at 1000 V s^{-1}, only a few percent of the molecules are optimally docked with the modified electrode surface and ready to transfer an electron, while at slowest scan rates all the tethered molecules have time to achieve the active configuration. This attenuation with scan rate is much greater than observed for protein molecules that are directly adsorbed on an electrode by stable non-covalent interactions [27]. The length and nature of the 'rope' will be important, as suggested by experiments with azurin molecules connected together by a linker [33]. Structure prediction algorithms for the linker sequence of c555m (Pôle Bio-Informatique Lyonnais for Network Protein Sequence Analysis) suggest there are two stretches having a helical conformation. These are highlighted in gray in Fig. 1.

An additional effect that we cannot discount at this stage is that both proteins may form dimers [11] which are unable to carry out electron transfer with the electrode. The rate of electron transfer might then be governed by the dimer-to-monomer transition. Increasing salt concentration would shift the equilibrium towards the monomeric state, allowing for faster electron transfer.

The k_0 value of 14 000 s^{-1} corresponds to an ET half time ($t_{1/2}$) of approximately 50 μs at zero driving force. The kinetics lie in the physiological range: $t_{1/2}$ values of 40–60 μs have been measured for ET between the cytochrome *bc* complex, cytochrome c_y and the photosynthetic reaction center of *R. capsulatus* in vivo, involving ET to and from the cytochrome and shuttling between the two redox partners [7].

In conclusion, these results present encouraging prospects for future studies of the mechanisms and functional advantages of tethered ET proteins. *A. aeolicus* is of particular interest since highly homologous soluble and tethered proteins are both expressed in the same organism and direct comparison is possible, as shown in this work. This system can allow investigation of the characteristics of the linker sequence in a completely controlled system, offering novel insight into protein ET reactions and quantitative data on domain movements to establish the importance of flexibility in linker peptides [3,33]. To achieve this aim, further studies, including investigations of the effects of viscosity, are now underway. In addition to this particular system, wider applications and interesting new directions are now suggested, including using a tethered ET protein to transfer electrons to specific enzyme partners immobilized nearby, engineering a 'rope' onto otherwise soluble proteins to enhance electroactivity, and using a 'rope' to explore distance and diffusion effects.

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