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# Gated electron transfer of cytochrome $c_6$ at biomimetic interfaces: a time-resolved SERR study†

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The electron shuttle heme protein Cyt- $c_6$  from the photosynthetic cyanobacterium *Nostoc* sp. PCC 7119 was immobilized on nanostructured Ag electrodes coated with SAMs that mimic different possible interactions with its natural reaction partner PSI. The structure, redox potential, and electron-transfer dynamics of the SAM–Cyt- $c_6$  complexes were investigated by TR-SERR spectroelectrochemistry. It is shown that the heterogeneous electron-transfer process is gated both in electrostatic and hydrophobic–hydrophilic complexes. At long tunneling distances, the reaction rate is controlled by the tunneling probability, while at shorter distances or higher driving forces, protein dynamics becomes the rate-limiting event.

## Introduction

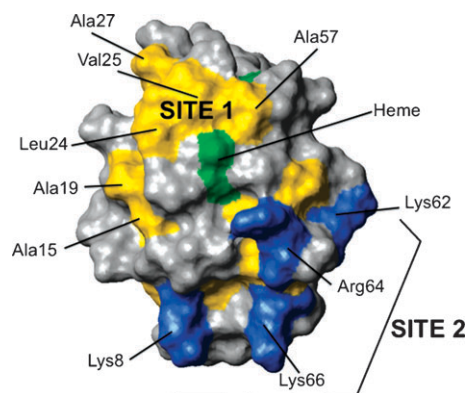
Cytochrome  $c_6$  (Cyt- $c_6$ ) is a monomeric soluble heme protein found in photosynthetic organisms, such as some cyanobacteria and green algae. Its natural function is transporting electrons up taken from the  $b_6f$  membrane-bound complex to photosystem I (PSI).<sup>1</sup> In plants, this role is exclusively played by the copper protein plastocyanin (Pc), but several lower organisms have the ability to produce either Pc or Cyt- $c_6$  depending on the bioavailability of Cu and Fe.<sup>2</sup> This is the case of the cyanobacterium *Nostoc* (formerly *Anabaena*) PCC 7119 (hereinafter *Nostoc*).

Cyt- $c_6$  from *Nostoc* is a basic protein (pI  $\approx$  9) with a molecular mass of 9.7 kDa.<sup>3</sup> Although its three-dimensional structure has not been resolved, sequence homology and spectroscopic evidence indicate that the heme iron is coordinated by histidine 19 and methionine 58 as axial ligands, yielding a six-coordinated low spin (6c-LS) heme within the pH range 5–11.<sup>3</sup>

Transient absorption spectroscopy shows that *in vitro* inter-protein electron transfer (ET) from *Nostoc* Cyt- $c_6$  to PSI follows biphasic kinetics. These results were interpreted in

terms of a gated ET mechanism.<sup>4–6</sup> Structural modeling of Cyt- $c_6$ , as well as kinetic experiments with point mutants, suggests the existence of two areas on the protein surface responsible for the interactions with PSI. A basic patch defined by residues Lys62, Lys66, and possibly Arg64 is believed to provide the long-range electrostatic interactions that drive the formation of the initial interprotein complex. It is hypothesized that docking through this site fails to provide an efficient electron pathway and, thus, transient rearrangement of the complex is required for efficient electron transfer to take place. The hydrophobic patch located in close vicinity to the partially exposed heme edge is considered essential for establishing the optimal electron pathway (Fig. 1).<sup>4,7,8</sup>

On the other hand, NMR investigations of the transient complex between Cyt- $c_6$  and the Cyt- $f$  soluble truncated subunit of the  $b_6f$  complex from *Nostoc* indicate that the interprotein contact region is mainly comprised of non-polar



**Fig. 1** Functional areas of *Nostoc* cytochrome Cyt- $c_6$ . Non-polar residues at site 1 are marked in yellow, positively charged residues at site 2 are in blue, and the heme group is in green. The surface map corresponds to the lowest energy conformer inferred from the NMR protein structure.<sup>9,10</sup>

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residues (ca. 61%).<sup>9</sup> Similar studies on Cyt-*c*<sub>6</sub>-PSI complexes reveal that both basic and non-polar amino acids are involved in the interactions.<sup>10</sup> More recently, CW-EPR and ENDOR experiments demonstrated the coexistence of two LS ferric forms, one of them highly anisotropic. The finding was related to the need for structural flexibility of Cyt-*c*<sub>6</sub> in its interactions with the two different redox partners.<sup>11,12</sup>

In this work, we present a study of the interactions of *Nostoc* Cyt-*c*<sub>6</sub> with biomimetic surfaces having different electrostatic and hydrophobic properties, and the impact of these interactions on the dynamics of the ET reaction. Besides the wild type (WT) protein, we have studied the mutant M58H, in which the sixth axial ligand methionine has been replaced by a histidine to obtain a bis-histidine coordination.

The model systems consist of nanostructured Ag electrodes coated by self-assembled monolayers (SAMs) of alkanethiols having varying chain lengths and terminal functionalities.<sup>13</sup> Adsorption and ET dynamics are monitored by stationary and time-resolved (TR) surface-enhanced resonance Raman (SERR) spectroelectrochemistry.<sup>14</sup>

## Experimental

### Chemicals

16-Mercaptohexadecanoic acid (C<sub>15</sub>-COOH), 11-mercapto-undecanoic acid (C<sub>11</sub>-COOH), 11-mercapto-1-undecanol (C<sub>11</sub>-OH), 1-mercaptododecane (C<sub>11</sub>-CH<sub>3</sub>), sucrose, potassium chloride, potassium sulfate, and potassium phosphate were purchased from Sigma-Aldrich and used without further purification. 16-Mercaptohexadecanol (C<sub>16</sub>-OH) was synthesized as described previously.<sup>15,16</sup> 1-Mercaptoheptadecane (C<sub>16</sub>-CH<sub>3</sub>) was prepared from the corresponding bromide by nucleophilic displacement with thioacetate followed by basic deprotection and standard purification methods.

Water used in all experiments was purified by a Millipore system and its resistance was more than 18 MΩ.

### Protein expression and purification

Wild type cytochrome *c*<sub>6</sub> and its M58H mutant from *Nostoc* sp. PCC7119 were expressed under aerobic conditions in *Escherichia coli* MC1061-transformed cells and purified as described elsewhere.<sup>8</sup> The purity of the protein preparations was greater than 95% as judged from the ratio of absorbance at 553 and 280 nm for reduced Cyt-*c*<sub>6</sub> and from the absence of other visible bands on Coomassie-stained SDS-PAGE. Protein concentrations were determined using an extinction coefficient  $\epsilon_{553} = 26.2 \text{ mM}^{-1} \text{ cm}^{-1}$  for reduced Cyt-*c*<sub>6</sub>.<sup>3</sup>

### Redox titrations

The redox potential value in solution for the M58H mutant was determined by UV-Vis potentiometric titrations as previously described,<sup>3,17</sup> by following absorbance changes at 554 minus 570 nm. Errors of the experimental determinations were less than 10 mV.

### Resonance Raman

Protein samples were placed inside a cylindrical quartz cell that is rotated at ca. 5 Hz to avoid laser-induced degradation.

Spectra were measured at room temperature in back-scattering geometry using a confocal microscope coupled to a single stage spectrograph (Jobin Yvon, LabRam 800 HR) equipped with a 2400 lines per mm grating and a liquid-nitrogen-cooled back-illuminated CCD detector. Elastic scattering was rejected by a Notch filter. All spectra reported here were measured with the 413 nm line of a cw krypton ion laser (Coherent Innova 300 c) focused on the sample by means of a long working distance objective (20×; N.A. 0.35). Typically, experiments were performed with laser powers of ca. 5 mW at the sample. Effective acquisition times were ca. 10–20 s with an increment per data point of 0.57 cm<sup>-1</sup>.

### Electrode modification

Silver-ring electrodes were mechanically polished with 3 M polishing films from 30 to 1 micron grade. After washing, electrodes were subjected to oxidation–reduction cycles in 0.1 M KCl to create a SER-active nanostructured surface. Subsequently, the electrodes were incubated in 1 mM ethanolic solutions of the alkanethiols for 24 h, rinsed, and transferred to the spectroelectrochemical cell.

### Surface-enhanced resonance Raman

The spectroelectrochemical cell for SERR measurements has been described elsewhere.<sup>18</sup> Briefly, a Pt wire and a Ag/AgCl electrode are used as counter and reference electrodes, respectively. All potentials cited in this work refer to the Ag/AgCl (3 M KCl) electrode. The working electrode is an 8 mm diameter and 2.5 mm height silver ring mounted on a shaft that is rotated at ca. 5 Hz to avoid laser-induced sample degradation. The electrolyte solution (12.5 mM phosphate buffer, pH = 7.0; 12.5 mM K<sub>2</sub>SO<sub>4</sub>) was bubbled prior to the measurements with catalytically purified oxygen-free argon, and Ar overpressure was maintained during the experiments. Protein was added to the electrochemical cell from a stock solution to form a 0.3 μM solution and incubated at room temperature for ca. 15 min before starting the experiments. SERR spectra were measured in back-scattering geometry as described in the previous section. Typically, experiments were performed with laser powers of 2.5–5 mW at the sample, effective acquisition times between 1 and 30 s, and increments per data point of 0.57 cm<sup>-1</sup>. For TR SERR experiments, potential jumps of variable height and duration were applied to trigger the reaction. SERR spectra were measured at variable delay times after each jump. Synchronization of potential jumps and measuring laser pulses was achieved with a home-made four-channel pulse-delay generator. The measuring pulses were generated by passing the cw laser beam through two consecutive laser-intensity modulators (Linos), which give a total extinction better than 1 : 50 000 and a time response of ca. 20 ns.

After background subtraction, the spectra were treated by component analysis in which the spectra of the individual species were fitted to the measured spectra using home-made analysis software. All experiments were repeated several times to ensure reproducibility.



## Kinetic simulations

Kinetic analysis was performed using GNU octave software. The set of differential equations that describe the kinetics for the proposed reaction mechanism was solved numerically, yielding concentration profiles for the four chemical species involved. Five adjustable parameters ( $A$ ,  $\beta$ ,  $\lambda$ ,  $k_1$ , and  $k_2$ ; *vide infra*) were varied until minimizing the standard deviations with respect to the experimental data. In this procedure, the experimental data set corresponds to a large number of experiments that include different chain lengths of the SAMs and various driving forces and solution viscosities. Further details are given in the following sections.

## Results and discussion

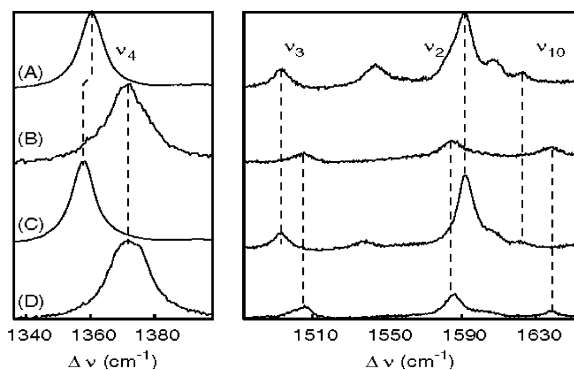
### Resonance Raman spectroscopy in solution

Wild type Cyt- $c_6$  and its M58H mutant were first characterized by RR spectroscopy in solution, *i.e.* under conditions that guarantee preservation of the native structures, as a prerequisite for the subsequent studies of the immobilized proteins.

Assuming an idealized  $D_{4h}$  symmetry for the porphyrin chromophore, resonance enhancement of heme proteins is only expected for the *gerade* in-plane vibrational modes. Specifically, under Soret-band excitation, RR spectra are dominated by the totally symmetric modes  $A_{1g}$ , which gain intensity *via* the A-term enhancement mechanism.<sup>19</sup> Due to the lower symmetry that characterizes actual hemes, also non-totally symmetric modes  $B_{1g}$ ,  $A_{2g}$ , and  $B_{2g}$  gain intensity, but to a lesser extent.<sup>20,21</sup> RR spectra of ferrous and ferric WT-Cyt- $c_6$  in the so-called marker band region (*ca.* 1300–1700  $\text{cm}^{-1}$ )<sup>22</sup> are shown in Fig. 2.

Vibrational modes observed in this spectral region are largely derived from C–C and C–N stretching vibrations of the porphyrin. Due to the increased electron back donation of  $\text{Fe}^{2+}$  compared to  $\text{Fe}^{3+}$  into the  $\pi^*$  orbital of the porphyrin, which weakens the C–N bonds, most bands are significantly up-shifted upon oxidation. Moreover, they are also sensitive markers of the spin and ligation pattern of the heme iron.<sup>13,14</sup>

Band positions, shapes (Lorentzian), widths, and relative intensities observed in the RR spectra of ferrous and ferric WT-Cyt- $c_6$  are characteristic of 6c-LS type *c* hemes with the



**Fig. 2** RR spectra of: (A) ferrous WT-Cyt- $c_6$ , (B) ferric WT-Cyt- $c_6$ , (C) ferrous M58H-Cyt- $c_6$ , and (D) ferric M58H-Cyt- $c_6$ . All measurements were performed with 413 nm laser excitation. Bands are labeled according to Hu *et al.*<sup>21</sup>

Met–His axial coordination pattern,<sup>13,23,24</sup> as predicted from the sequence and in analogy to equivalent proteins from other organisms.<sup>3,4,25</sup>

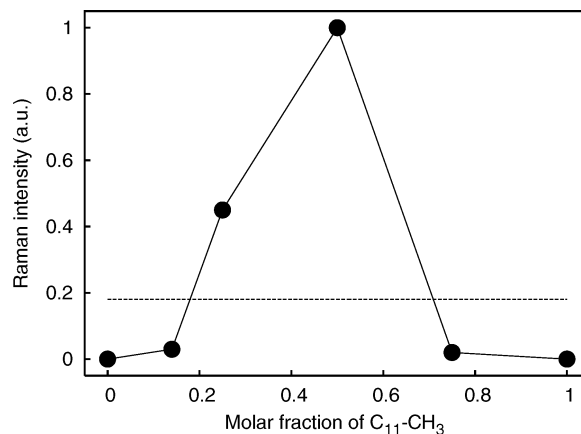
For the M58H mutant, RR spectra exhibit only slight shifts of most bands with respect to the WT protein. For example, in the reduced form, the most prominent band  $\nu_4$  appears at 1358  $\text{cm}^{-1}$  for the mutant compared to 1360  $\text{cm}^{-1}$  for the WT. These small spectral changes are compatible with the axial-ligand substitution of methionine by histidine as neither the spin state nor the coordination number of the heme iron are changed.<sup>13,23,24</sup> Complete sets of spectral parameters are given in ESI†.

RR intensities are proportional to the concentration of the species. Proportionality factors ( $F$ ) for the reduced and oxidized proteins were determined from the intensity ratios reduced–oxidized of RR spectra measured under identical conditions, *i.e.*, adopting  $F = 1$  for the ferric form. The value of 0.143 obtained for both ferrous forms (WT and M58H) is used in the subsequent sections for quantification of the redox equilibria and dynamics.

### Protein adsorption and redox equilibria

It has been proposed that Cyt- $c_6$  employs two distinct sites for the interaction with its redox partner PSI: a positively charged domain responsible for efficient docking and a hydrophobic patch that provides an optimized but transient ET pathway (see Introduction). With these premises in mind, we have investigated the immobilization of Cyt- $c_6$  on nanostructured Ag electrodes coated with uncharged SAMs of variable hydrophobicity as well as negatively charged SAMs. Protein adsorption was monitored by SERR under Soret-band excitation, *i.e.* under conditions that ensure sensitive and selective detection solely of Cyt- $c_6$  adsorbed molecules. Fig. 3 shows normalized SERR intensities measured for Cyt- $c_6$  adsorbed on mixed SAMs composed of variable ratios of 1-mercaptododecane and 11-mercapto-1-undecanol ( $\text{C}_{11}\text{-CH}_3$  and  $\text{C}_{11}\text{-OH}$ , respectively).

Optimal adsorption is achieved for compositions around 1 : 1, while no adsorption is observed for SAMs of the pure



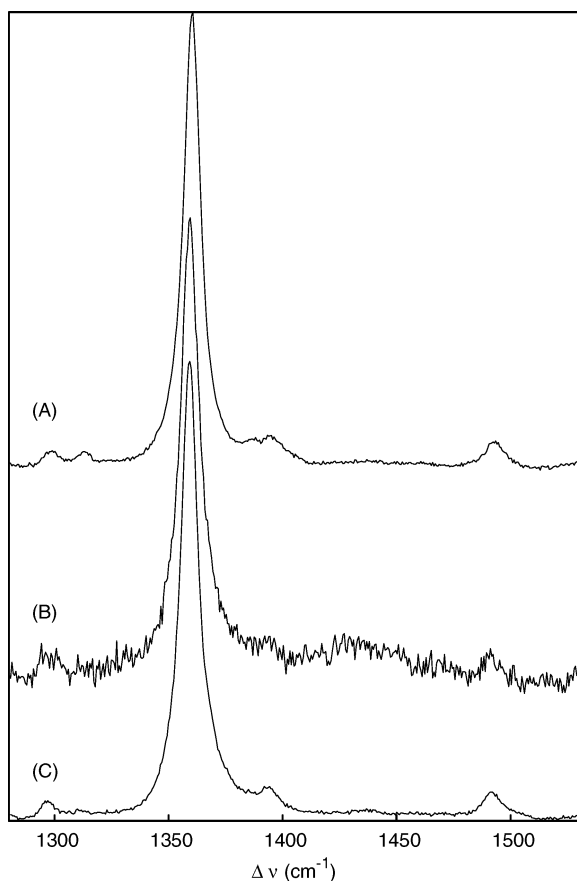
**Fig. 3** Normalized SERR intensity of WT-Cyt- $c_6$  adsorbed on  $\text{C}_{11}\text{-CH}_3/\text{C}_{11}\text{-OH}$  mixed SAMs of variable composition (circles and solid line). The dashed line represents the normalized SERR intensity obtained for WT-Cyt- $c_6$  adsorbed on a  $\text{C}_{10}\text{-COOH}$  pure SAM.

alkanethiol components. Note that the compositions indicated in Fig. 3 correspond to the solution compositions utilized for self-assembly and not to the final surface compositions.<sup>26</sup>

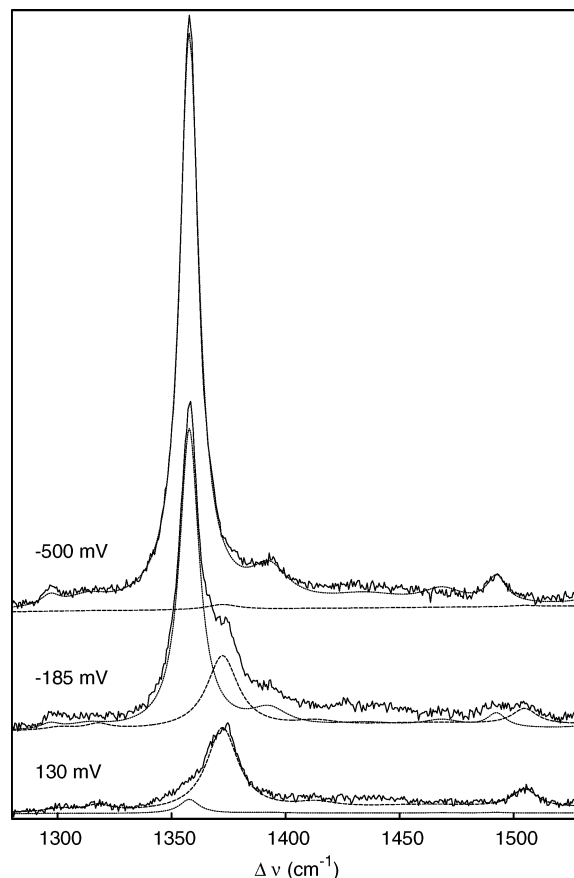
For a negatively charged SAM of comparable length composed of 11-mercaptopundecanoic acid (C<sub>10</sub>-COOH), the amount of adsorbed protein is about one-fifth of the maximum value obtained for C<sub>11</sub>-CH<sub>3</sub>/C<sub>11</sub>-OH mixed SAMs. At pH 7.0, employed in all the studies reported here, the degree of ionization of the C<sub>10</sub>-COOH SAMs is about 10%.<sup>27</sup> These results indicate that optimal adsorption of Cyt-*c*<sub>6</sub> requires an adequate balance of hydrophobic and hydrophilic interactions, while electrostatic forces lead to relatively weak adsorption.

This behavior is in contrast to horse heart and yeast cytochromes *c*, which have significantly larger dipole moments and better defined positive patches around the partially exposed heme,<sup>13,14,28–30</sup> but resemble that of cytochrome *c*<sub>552</sub> which exhibits a hydrophobic binding domain surrounding the heme pocket.<sup>15,16</sup>

For both modes of immobilization, the native structure of Cyt-*c*<sub>6</sub> is retained, at least at the level of the active site. This is inferred from the SERR spectra of the adsorbed protein, which are essentially identical to the corresponding RR spectra of the native protein in solution (Fig. 4).



**Fig. 4** Comparison of the RR spectrum of dithionite-reduced WT-Cyt-*c*<sub>6</sub> in solution (A) and SERR spectra of the electrochemically reduced protein adsorbed on C<sub>15</sub>-COOH (B) and on C<sub>16</sub>-CH<sub>3</sub>/C<sub>16</sub>-OH (C) SAMs in the spectral region of the  $\nu_3$  and  $\nu_4$  modes.



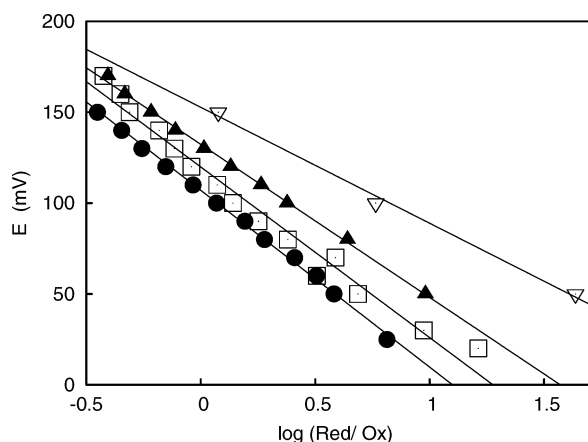
**Fig. 5** SERR spectra of M58H-Cyt-*c*<sub>6</sub> adsorbed on C<sub>10</sub>-COOH measured at various potentials. The spectral components of the reduced and oxidized protein are represented with dotted and dashed lines, respectively.

The adsorbed protein sensitively responds to the electrode potential, as evidenced by the potential-dependent spectral changes. For C<sub>11</sub>-CH<sub>3</sub>/C<sub>11</sub>-OH and C<sub>16</sub>-CH<sub>3</sub>/C<sub>16</sub>-OH mixed SAMs, SERR spectra can be simulated by summation of the RR component spectra of ferrous and ferric Cyt-*c*<sub>6</sub> in different proportions (Fig. 5), confirming the structural integrity of the adsorbed protein in the entire potential range explored.

In the case of C<sub>15</sub>-COOH SAMs, in order to obtain a perfect reproduction of the SERR spectra at the various potentials, it is necessary to incorporate two potential-independent weak Gaussian bands at *ca.* 1349 and 1372 cm<sup>-1</sup>, in addition to the Lorentzian component spectra of ferrous and ferric native Cyt-*c*<sub>6</sub> determined from the RR spectra in solution. The origin of the Gaussian bands is unclear, but the shape and potential-independence suggest that it represents a fraction of the electrostatically adsorbed protein that is redox inactive. Similar results were obtained both for WT- and M58H-Cyt-*c*<sub>6</sub>.

From the component spectra, and by using the proportionality factors determined from the RR spectra in solution (*vide supra*), one can determine the relative surface concentrations of reduced and oxidized Cyt-*c*<sub>6</sub>, and subsequently the standard reduction potentials through the Nernst equation (eqn (1)):

$$E = E^0 - \frac{RT}{nF} \ln \frac{[\text{Cyt}^{2+}]}{[\text{Cyt}^{3+}]} = E^0 - \frac{RT}{nF} \ln \frac{(I \times F)_{\text{Cyt}^{2+}}}{(I \times F)_{\text{Cyt}^{3+}}} \quad (1)$$



**Fig. 6** Nernst plots of WT-Cyt-*c*<sub>6</sub> adsorbed on different SAMs. Circles: C<sub>10</sub>-COOH. Squares: C<sub>15</sub>-COOH. Up-triangles: C<sub>11</sub>-CH<sub>3</sub>/C<sub>11</sub>-OH. Down-triangles: C<sub>16</sub>-CH<sub>3</sub>/C<sub>16</sub>-OH.

**Table 1** Standard reduction potentials ( $E^0$ ) and number of transferred electrons ( $n$ ) obtained for WT- and M58H-Cyt-*c*<sub>6</sub> under the different immobilization conditions

SAM	WT-Cyt- <i>c</i> <sub>6</sub> $E^0$ /mV ( $n$ )	M58H-Cyt- <i>c</i> <sub>6</sub> $E^0$ /mV ( $n$ )
C <sub>10</sub> -COOH	114 (0.7)	-185 (0.7)
C <sub>15</sub> -COOH	108 (0.7)	-199 (0.8)
C <sub>11</sub> -CH <sub>3</sub> /C <sub>11</sub> -OH	150 (0.9)	n.d.
C <sub>16</sub> -CH <sub>3</sub> /C <sub>16</sub> -OH	133 (0.8)	n.d.
Solution	<sup>a</sup> 140	-140

<sup>a</sup> Taken from Molina-Heredia *et al.*<sup>3</sup>

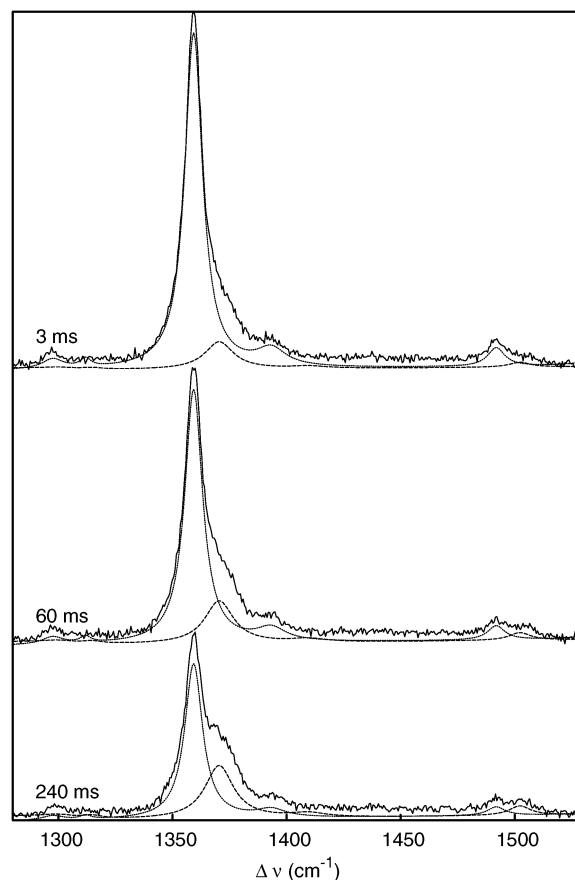
where  $I$  and  $F$  represent the relative intensities of the spectral components and their proportionality factors, respectively. The symbols  $R$  and  $T$  have their usual meaning, and  $n$  is the number of transferred electrons. Fig. 6 shows representative Nernst plots, and the results are summarized in Table 1.

Within experimental error, the  $E^0$  values determined for WT-Cyt-*c*<sub>6</sub> adsorbed on mixed SAMs are similar to the one reported for the protein in solution,<sup>3</sup> and the calculated  $n$  factors are close to the ideal value of 1.

In the case of electrostatic adsorption, the determined  $E^0$  values, both for WT and M58H Cyt-*c*<sub>6</sub>, are negatively shifted with respect to the values in solution. The shift is most likely due to the interfacial potential drop, which is expected to be more severe at charged SAMs.<sup>27,31</sup> Also, the  $n$  values are slightly lower compared to the uncharged SAMs, reflecting a larger heterogeneity of the electrostatically adsorbed protein. Note that, in contrast to conventional electrochemical methods, SERR probes all the adsorbed species including those that are electrochemically inactive.

### Electron-transfer dynamics

The dynamics of the heterogeneous ET reactions of WT and M58H Cyt-*c*<sub>6</sub> immobilized on different SAM-coated electrodes was investigated by TR-SERR. In these experiments, the redox equilibrium of the adsorbed protein is perturbed by applying a potential jump from an initial potential  $E_i$  to a final potential  $E_f$ . SERR spectra are measured at variable delay



**Fig. 7** TR-SERR spectra of WT-Cyt-*c*<sub>6</sub> adsorbed on a C<sub>16</sub>-CH<sub>3</sub>/C<sub>16</sub>-OH SAM, measured at different delay times.

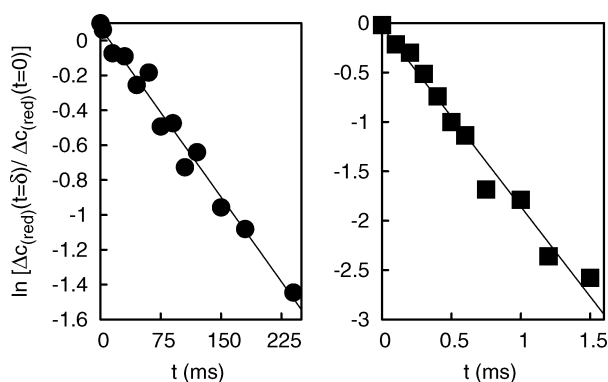
times with respect to the potential jump and analyzed in terms of ferric and ferrous Cyt-*c*<sub>6</sub> spectral components as described above. Typical TR-SERR spectra are shown in Fig. 7.

In a first approach, one may assume that the perturbed system will evolve toward the equilibrium value at the final potential following first-order kinetics with an apparent ET rate constant  $k_{ET}^{app}$ . Thus, the one-step relaxation process can be described according to eqn (2):

$$\ln \frac{[\text{Cyt}^{2+}]_{E_f} - [\text{Cyt}^{2+}]_I}{[\text{Cyt}^{2+}]_{E_f} - [\text{Cyt}^{2+}]_{E_i}} = \ln \frac{\Delta C_t}{\Delta C_0} = -k_{ET}^{app} t \quad (2)$$

where subscripts  $E_f$  and  $E_i$  denote the equilibrium concentrations of the reduced protein at the final and initial potentials, respectively, and  $t$  is the probing delay time with respect to the applied potential jump. As shown in Fig. 8 for some representative cases, plots of the experimental data according to eqn (2) are linear within the experimental error, thus justifying the one-step relaxation assumption. The  $k_{ET}^{app}$  values obtained in this way for different experimental conditions, including different overpotentials ( $\eta$ ), are summarized in Table 2.

First, we analyze the distance dependence of  $k_{ET}^{app}$  measured at zero driving force, *i.e.* for  $E_f = E^0$  ( $\eta = 0$  mV). As shown in Fig. 9, the variation of  $k_{ET}^{app}(0)$  for Cyt-*c*<sub>6</sub> with the length of the SAM is qualitatively similar to the dependencies observed for mammalian cytochrome *c* electrostatically adsorbed on



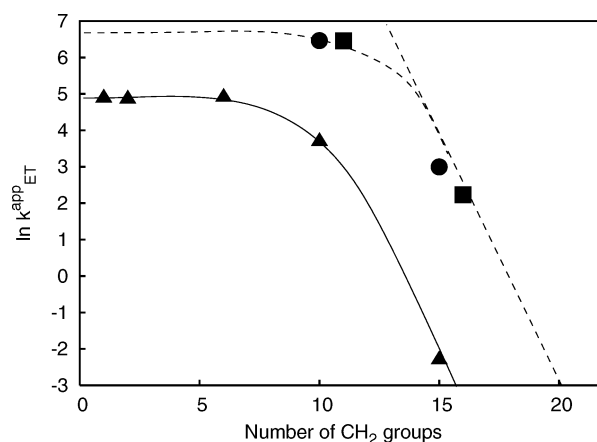
**Fig. 8** Reduction kinetics of Cyt-*c*<sub>6</sub> under various experimental conditions. Circles: WT-Cyt-*c*<sub>6</sub> on a C<sub>16</sub>-CH<sub>3</sub>/C<sub>16</sub>-OH SAM;  $\eta = 0$  mV. Squares: M58H-Cyt-*c*<sub>6</sub> on a C<sub>10</sub>-COOH SAM;  $\eta = -275$  mV.

carboxyl-terminated SAMs and for other proteins immobilized on different SAM-coated electrodes.<sup>28,32–38</sup>

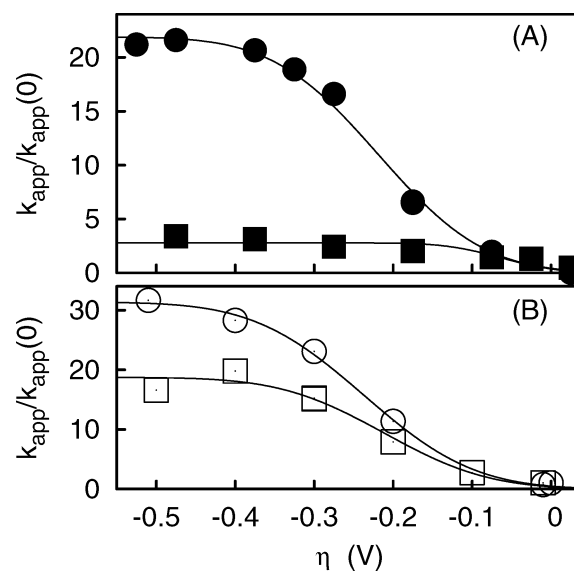
At long distances,  $k_{ET}^{app}(0)$  increases exponentially upon shortening the length of the SAM, as expected for a non-adiabatic ET mechanism,<sup>28,32–38</sup> with a decay factor  $\beta \approx 1.1$  per methylene group.

For thinner SAMs, however, the reaction rate becomes insensitive to the distance, reaching a plateau. The values determined for Cyt-*c*<sub>6</sub> display a very similar distance dependence for both charged and uncharged SAMs and are similar at each SAM thickness for zero driving force. However, the rate constants are significantly larger than for mammalian cytochrome *c* electrostatically adsorbed. The first observation is quite surprising taking into account that the protein is likely to adsorb *via* different residues to the different surfaces, and that relatively small changes of orientation are expected to result in significant variations of the electronic coupling.<sup>30</sup> On the other hand, the fact that ET rates for Cyt-*c*<sub>6</sub> are much larger than for mammalian cytochrome *c* is most likely the result of a stronger electronic coupling rather than a smaller reorganization energy, as both proteins share identical redox centers and quite similar heme pockets.

For the thinner SAMs studied (C<sub>10</sub>-COOH and C<sub>11</sub>-CH<sub>3</sub>/C<sub>11</sub>-OH),  $k_{ET}^{app}(0)$  drops by *ca.* a factor of 2 upon a 50% increase of the solution viscosity ( $\rho$ ). In contrast, no viscosity effect is observed for C<sub>15</sub>-COOH and C<sub>16</sub>-CH<sub>3</sub>/C<sub>16</sub>-OH, both for WT and M58H Cyt-*c*<sub>6</sub> (Table 2). Consistently,  $k_{ET}^{app}(\eta)$  shows a clear increase with the applied overpotential ( $\eta$ ) for



**Fig. 9** Semi-logarithmic plots of apparent ET rate constants as a function of the number of methylene groups of the spacers. Triangles: horse heart cytochrome *c* on C<sub>*x*</sub>-COOH SAMs. Squares: WT-Cyt-*c*<sub>6</sub> on C<sub>*x*</sub>-CH<sub>3</sub>/C<sub>*x*</sub>-OH SAMs. Circles: WT-Cyt-*c*<sub>6</sub> on C<sub>*x*</sub>-COOH SAMs. All values were obtained at room temperature with  $\eta = 0$  mV and  $\rho = 1$  cP.



**Fig. 10** Normalized apparent ET rate constants as a function of the applied overpotential. (A) M58H-Cyt-*c*<sub>6</sub> on C<sub>15</sub>-COOH (filled circles) and on C<sub>10</sub>-COOH (filled squares) SAMs at 1 cP. (B) WT-Cyt-*c*<sub>6</sub> on a C<sub>15</sub>-COOH SAM at 1 cP (empty circles) and 1.5 cP (empty squares).

**Table 2** Apparent ET rate constants obtained for WT- and M58H-Cyt-*c*<sub>6</sub> under various experimental conditions

SAM	$\eta$ /mV	$k_{ET}^{app}/s^{-1}$		$k_{ET}^{app}/s^{-1}$	
		WT-Cyt- <i>c</i> <sub>6</sub>		M58H-Cyt- <i>c</i> <sub>6</sub>	
		$\rho = 1$ cP	$\rho = 1.5$ cP	$\rho = 1$ cP	$\rho = 1.5$ cP
C <sub>15</sub> -COOH	0	20	19	66	58
C <sub>15</sub> -COOH	-500	639	359	1475	259
C <sub>10</sub> -COOH	0	639	302	854	419
C <sub>10</sub> -COOH	-500	1230	683	2568	1040
C <sub>16</sub> -CH <sub>3</sub> /C <sub>16</sub> -OH	0	9.3	8.8	n.d.	n.d.
C <sub>11</sub> -CH <sub>3</sub> /C <sub>11</sub> -OH	0	637	266	n.d.	n.d.



the thicker SAMs, but nearly no change for the thinner ones (Fig. 10A).

These results suggest that at longer distances, the rate of the redox reaction is controlled by tunneling probability, while at shorter ones, a viscosity-dependent process becomes rate limiting. Note, however, that this conclusion appears to be valid only at very low driving forces. For overpotentials higher than 100 mV, an increasing viscosity effect is also observed for the thicker SAMs, reaching a  $k_{\text{ET}}^{\text{app}}(\eta)/k_{\text{ET}}^{\text{app}}(0)$  ratio of *ca.* 1.7 in the plateau region (Fig. 10B).

### Kinetic modeling

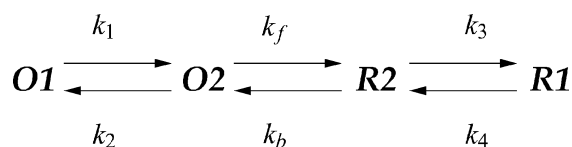
The results described in the preceding sections are consistent with a reaction scheme in which the measured reaction rate is a convolution of protein dynamics and tunneling probabilities, as recently demonstrated for mammalian cytochrome *c*.<sup>20,30</sup> Within this context, the simplest possible reaction mechanism that can be proposed is a two-state model,<sup>33,39</sup> (Scheme 1) which is also consistent with kinetic studies of Cyt-*c*<sub>6</sub>/PSI interprotein complexes.<sup>5,6,8</sup>

In this model, it is assumed that ferric Cyt-*c*<sub>6</sub> adsorbs on the electrode surface in a thermodynamically stable orientation (O1), which does not establish an efficient electron pathway and, thus, can be regarded as redox inactive. O1 reorients to the redox-active form O2 with a rate constant  $k_1$  and, in this configuration, accepts an electron from the electrode with a rate constant  $k_f$ . The product R1 can then reorient back to the redox-inactive form of the ferrous protein R2 ( $k_3$ ). Species O1 and O2 on one hand, and R1 and R2 on the other are spectroscopically undistinguishable as they only differ in orientation.<sup>20</sup>

The consistency of this simplified kinetic model with the experimental results was tested numerically using a number of approximations. First, it is assumed that the surface binding energies of the four species are equal. This assumption implies that redox-linked structural changes are not significant, and that the short-range forces involved in protein binding do not depend on the oxidation state of the heme iron.

These assumptions imply  $k_1 = k_4$  and  $k_2 = k_3$ . Second, the rate of rearrangement is considered to be independent of the SAM thickness and of the applied overpotential and is assumed to be influenced only by the medium viscosity.

The rate of diabatic heterogeneous electron transfer at a metal electrode can be expressed in terms of the high temperature limit of the Marcus semiclassical equation by integrating over all the electronic levels of the metal contributing to the process.<sup>40</sup> The expression can be simplified by approximating the Fermi distribution law as a step function<sup>41</sup> and relating the



**Scheme 1** Minimal kinetic model for the heterogeneous ET reaction of Cyt-*c*<sub>6</sub> adsorbed on SAM-coated electrodes. O and R denote oxidized and reduced protein, respectively. The numbers 1 and 2 refer to the redox-inactive and redox-active orientations, respectively.

rate constants for the forward and backward ET reactions through the Nernst equation:

$$k_f(0) = A \exp(-d\beta) \quad (3)$$

$$k_f(\eta) = k_f(0) \frac{1 - \text{erf}[(F\eta + \lambda)/(2\sqrt{\lambda RT})]}{1 - \text{erf}[\lambda/(2\sqrt{\lambda RT})]} \quad (4)$$

$$k_b(\eta) = k_f(\eta)/10^{-\eta/59} \text{ mV} \quad (5)$$

where  $\beta$  is the tunneling decay constant,  $d$  is the distance,  $A$  is a pre-exponential factor,  $\lambda$  is the reorganization energy, and the other symbols have their usual meaning.

For each viscosity value (1 and 1.5 cP), entire data sets that include various driving forces and SAM lengths were simulated using  $A$ ,  $\beta$ ,  $\lambda$ ,  $k_1$ , and  $k_2$  as adjustable parameters. As shown in the ESI†, good fits to all experimental data recorded under various conditions are obtained for values of  $A$ ,  $\beta$ , and  $\lambda$  of  $1.85 \times 10^{-12} \text{ s}^{-1}$ , 1.07 per methylene group, and 0.35 eV, respectively. The three values are quite reasonable and are in excellent agreement with previous reports on similar redox proteins,<sup>14,28,33–38</sup> thus confirming that the adopted kinetic model and its underlying assumptions are justified. For a viscosity of 1 cP, the best simulations are obtained with  $k_1 = 840 \text{ s}^{-1}$  and  $k_2 = 0.5 \text{ s}^{-1}$ . Both values decrease at 1.5 cP to 540 and  $0.2 \text{ s}^{-1}$ , respectively. Some representative simulations are shown in the ESI†.

### Conclusions

The present results show that Cyt-*c*<sub>6</sub> can efficiently adsorb both on neutral and on negatively charged surfaces that are designed for mimicking the essential features of the interactions with its natural reaction partner PSI. SERR experiments demonstrate that the adsorbed protein retains the native structure in two types of complexes. However, in contrast to previous predictions,<sup>4,7,8,25</sup> the highest affinity does not correspond to electrostatic complexes but to a balance of short-range hydrophobic–hydrophilic interactions.

TR-SERR experiments performed under various conditions, including variable SAM thicknesses, viscosities, and driving forces, demonstrate that the interfacial ET reaction follows a gated mechanism. The gating step implies a rearrangement from an electrochemically inactive configuration to another one that is optimized for ET, and is consistent with previous results for Cyt-*c*<sub>6</sub>-PSI complexes.<sup>4,7,8,25</sup> For long distances, the electron-tunneling probability is sufficiently low such that rearrangement of the complex is not rate limiting. The situation changes upon decreasing the thickness of the SAM spacer due the exponential increase of the ET rate and the relatively weak distance dependence of the rearrangement rate that, thus, becomes the rate-limiting step. Kinetic modeling of the entire data set under the different experimental conditions yields a consistent mechanistic picture with  $\lambda = 0.35 \text{ eV}$ .

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