Controlling Interfacial Electron-Transfer Kinetics of Cytochrome *c* with Mixed Self-Assembled Monolayers

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Prior work has established that self-assembled monolayers (SAMs) prepared with COOH-terminated alkanethiols ($HS(CH_2)_n$-COOH) can serve as excellent gold surface modifiers for the immobilization of horse cytochrome *c* (cyt *c*) in a stable electroactive state.[1,2] An important finding from these prior studies is that, for SAMs assembled using alkanethiols with $n \geq 8$, the standard electron-transfer rate constant ($k_{et}°$) depends exponentially on *n* and presumedly, therefore, on tunneling distance. The tunneling decay factor ($\beta = 1.0-1.1/CH_2$) obtained from these cyt *c* experiments is essentially identical to that obtained from small molecule electron-transfer (ET) studies at SAM-modified electrodes[3,4] and is consistent with a through-bond tunneling mechanism.

We have recently established the same quantitative dependence of ET rate vs distance for *Saccharomyces cerevisiae* (yeast) cyt *c* adsorbed on COOH SAM/Au substrates, i.e., $\beta = 1.0-1.1/CH_2$.[5] Although a similar β was expected, we were initially surprised to find large differences in $k_{et}°$ between yeast and horse cyt *c* when compared at identical SAMs. For yeast cyt *c*, $k_{et}°$ values were 10^2-10^3 smaller despite the fact that ET reorganization energies are similar for the two,[6] as are their tertiary structures and surface charge distributions.[7] For sure, these two cytochromes have substantial amino acid differences, 40 to be exact,[8] that hold the key to explaining this result. Mutagenesis studies are underway to test some single-site hypotheses in this regard, but these results are not the subject of the present communication.

We report here that interfacial ET rates of cytochromes *c* can be increased, dramatically in the case of the yeast species, by altering the structure of the SAM. Specifically, mixed monolayers have been prepared in which the COOH-thiol has been diluted through coassembly with OH-thiols. Numerous reports of mixed SAMs have appeared recently including protein adsorption studies.[3,9] We will show below that increases in ET rate of $\geq 10^3$ for yeast cyt *c* are possible when mixed COOH/OH SAMs are substituted for COOH SAMs of comparable thickness.

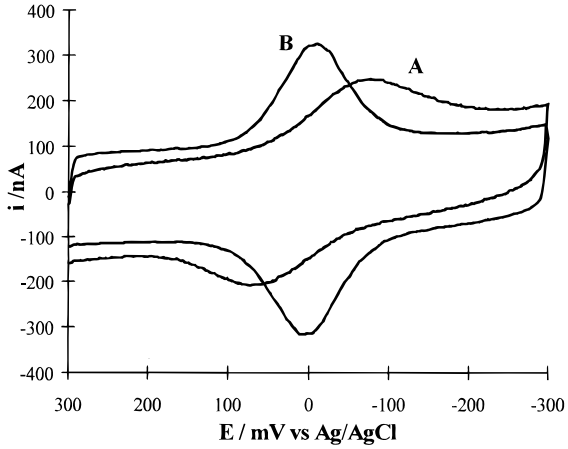

Figure 1. CVs of yeast cyt *c* (C102T) adsorbed on (A) a pure acid SAM prepared from $HS(CH_2)_{10}COOH$ and (B) a mixed SAM prepared from $HS(CH_2)_{10}COOH$ and $HS(CH_2)_7OH$. $\nu = 50$ mV/s. Electrode area = 0.32 cm^2. Solution: 22 mM potassium phosphate buffer, pH 7.0.

Experimental procedures followed prior protocols. Evaporated gold films (200 nm Au/10 nm Ti/glass; Evaporated Metal Films, Ithaca, NY) were pretreated chemically in hot HNO_3[1d] or electrochemically,[1c] with similar results. Mixed monolayers were self-assembled from 2.5 mM COOH-thiol and 2.5 mM OH-thiol in ethanol. Ex situ reflection FTIR spectroscopy revealed substantial fractions of both the OH and COOH functionalities. Substituted alkanethiols were synthesized according to literature precedent,[10] with characterization data available as Supporting Information. Yeast cyt *c* was the mutated iso-1 form in which the reactive cysteine at position 102 has been replaced by a benign threonine, i.e., the C102T variant.[11] C102T was isolated and purified from overexpressed *Escherichia coli*.[11] Horse cyt *c* (Sigma, Type VI) was chromatographically purified. Electrochemical impedance spectroscopy (EIS)[1c] and cyclic voltammetry (CV)[1b] were used to determine $k_{et}°$ at $E = E°'$, i.e., the standard ET rate constant.

CVs for yeast cyt *c* adsorbed on a $C_{10}COOH$ SAM and on a $C_{10}COOH/C_7OH$ mixed SAM are shown in Figure 1. The response for $C_{10}COOH$ is typical for a pure acid SAM,[5] with kinetic analysis yielding $k_{et}° = 0.21$ s^{-1}, which is ∼100-fold smaller than $k_{et}°$ for horse cyt *c* at an identical electrode. On $C_{10}COOH/C_7OH$, however, yeast cyt *c* ET is much faster, and in fact, the CV shown in Figure 1 is kinetically reversible on this time scale. EIS analysis yielded $k_{et}° = 500$ s^{-1}, a roughly 2500-fold increase over the $C_{10}COOH$ SAM. This increased rate cannot be explained by a decrease in effective ET distance due to the shorter OH-alkanethiol diluent because, for a full 3-methylene decrease, only a 20-fold rate increase is expected for $\beta = 1.0-1.1/CH_2$. Furthermore, charging currents and interfacial capacitances gave no evidence of any noteworthy decrease in SAM thickness upon inclusion of the C_7OH species. Indeed, the ET rate for yeast cyt *c* at this mixed SAM was ∼30 times faster than that obtained for a pure C_7COOH SAM ($k_{et}° = 18$ s^{-1}), which is definitely a thinner film. Last, faster ET rates ($k_{et}° = 2$ s^{-1}) were

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Table 1. Standard Electron-Transfer Rate Constants ($k_{et}°$, s^{-1}) for Cytochrome/SAM/Gold Monolayer Electrodes[a−c]

SAM structure[d]	horse cytochrome *c*	yeast cytochrome *c*
C_7COOH	1000	18
$C_{10}COOH$	60	0.21
$C_{10}COOH/C_7OH$		500
$C_{10}COOH/C_8OH$	300	400
$C_{10}COOH/C_{13}OH$		2.0

[a] Values of $k_{et}°$, measured using cyclic voltammetry and/or electrochemical impedance spectroscopy, are the ET rate constants at $E = E°'$, i.e., the condition of zero free energy driving force. Measurements were made at room temperature. [b] Percent relative standard deviation for $k_{et}°$ is ±10−30% based on electrode-to-electrode reproducibility. [c] Electroactive cyt *c* surface concentrations for all SAMs were in the range of 12−15 pmol/cm^2. [d] Designation represents alkanethiol structure, e.g., $C_{10}COOH$ refers to a SAM prepared using $HS(CH_2)_{10}COOH$.

measured even when yeast cyt *c* was adsorbed to a $C_{10}COOH/C_{13}OH$ mixed SAM, which is thicker than a $C_{10}COOH$ SAM ($k_{et}° = 0.21\ s^{-1}$) on the basis of capacitance.

Results for yeast and horse cyt *c* are collected in Table 1. Mixed SAMs result, too, in enhanced ET rates for horse cyt *c*, although the effect is noticeably weaker than for yeast. Also notable is that, for the $C_{10}COOH/C_8OH$ mixed SAM, yeast cyt *c* exhibits a faster ET rate than does horse, which is their same relative reactivity with yeast cyt *c* peroxidase (CCP).[12] Electroactive cyt *c* surface concentrations were fairly constant across all the SAM types, ranging from 12 to 15 pmol/cm^2.

The large increases in ET rate that result when COOH SAMs are replaced by mixed COOH/OH SAMs are proposed to result from enhanced electronic coupling at the yeast cyt *c*/SAM interface. The COOH SAM is clearly a much better surface for transmitting electrons to horse cyt *c* than to yeast cyt *c*. With mixed SAMs, however, electron transmission for both species improves, greatly in the case of yeast, so that rates become comparable. Some significant molecular differences must exist at these cytochrome/SAM interfaces to account for these results. Of key relevance is the report by Pelletier and Kraut, which described key differences at the protein−protein interfaces formed when CCP binds to each of the two cytochromes.[13] An X-ray structural analysis of cocrystals of each cytochrome with CCP revealed distinct modes of binding and possible ET routing. For the yeast CCP/yeast cyt *c* complex, binding appears to be primarily hydrophobic and a nonionic heme-to-heme ET pathway extending from CCP tryptophan-191 was proposed: Trp-191···Gly-192···Ala-193···Ala-194. The interior Trp-191 makes direct contact with the CCP heme, while Ala-194 appears to make direct contact with the recessed heme edge of yeast cyt *c*.[13] Support for this hypothesis has come from interprotein cross-linking and site-directed mutagenesis studies.[14−16] Conversely, analysis of the yeast CCP/horse cyt *c* cocrystal did not reveal a comparable ET pathway, and binding interactions were considerably more ionic.[13]

We propose that our results can be explained by the Pelletier-Kraut (PK) model for CCP/cyt *c* ET and, in turn, they constitute new electrochemical evidence in support of it. Apparently, when bound to a pure COOH SAM, yeast cyt *c* is unable to establish optimal binding and electronic coupling, presumedly due to inadequate hydrophobic interactions and the absence of a well-coupled ET pathway. We believe the operative pathway, by analogy to the PK model, would involve direct contact between the yeast cyt *c* heme edge and the terminal atom(s) of an alkanethiol. Inability to make this contact on a COOH SAM may be related to a lack of appropriate molecular texture and/or interfacial flexibility. An AFM study of COOH SAMs has characterized this surface as being "stiffer" than a methyl-terminated SAM.[17] Upon COOH dilution in a mixed SAM, however, we would expect the interface to exhibit a more irregular texture, a more hydrophobic character due to greater exposure of methylenes, and more conformational freedom.[18] The final result is an electrode interface that can bind yeast cyt *c* in an electronically well-coupled state, apparently by establishing direct molecular contact with the recessed heme edge.

Future work must consider more carefully the role played by the protonation state of the SAM. COOH SAMs exhibit apparent surface pK_a values of ∼8, which shift to more acidic values when coassembled with shorter alkanethiols.[19] We expect a similar situation for mixed COOH SAMs coassembled with shorter OH-alkanethiols. One could then argue that enhanced yeast cyt *c* ET at mixed SAMs arises primarily from stronger electrostatic binding due to the more extensive deprotonation of the mixed SAMs. The fact that $C_{10}COOH/C_{13}OH$ mixed SAMs give 10-fold faster ET rates for yeast cyt *c* than do $C_{10}COOH$ SAMs suggests, however, that that is not the case. Not only is $C_{10}COOH/C_{13}OH$ apparently a thicker SAM than $C_{10}COOH$ but its pK_a should be shifted alkaline because its carboxyl groups are more sheltered from the aqueous environment.[19] Finally, we note that horse cyt *c* transfers electrons quite well with COOH SAMs, with only fivefold rate enhancements resulting with mixed SAMs. Thus, although protonation of the SAM surface does change with monolayer structure, this phenomenon does not seem to be the dominant factor in explaining our results.

Surfaces of CCP and other ET proteins are irregularly textured, both chemically and topographically, including surface domains for binding protein partners. In retrospect, it does not seem too surprising that optimal artificial surfaces are likely to have similar features. Constant-chain-length COOH SAMs, for example, do mimic acidic protein surfaces but, as shown here, to an extent dictated by detailed structural features of the protein. We conclude that SAMs of constant chain length and low defect density are unlikely to be the surfaces of choice for binding ET proteins with optimal electronic coupling.

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Supporting Information Available: Experimental details (2 pages). See any current masthead page for ordering and Internet access instructions.

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