Published in final edited form as:

Europhys Lett. 2008 July ; 83(1): 180004. doi:10.1209/0295-5075/83/18004.

Evidence for protein conformational change at a Au(110)/protein interface

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Abstract

Evidence is presented that reflection anisotropy spectroscopy (RAS) can provide real-time measurements of conformational change in proteins induced by electron transfer reactions. A bacterial electron transferring flavoprotein (ETF) has been modified so as to adsorb on an Au(110) electrode and enable reversible electron transfer to the protein cofactor in the absence of mediators. Reversible changes are observed in the RAS of this protein that are interpreted as arising from conformational changes accompanying the transfer of electrons.

While there has been considerable progress in the determination of the structures of biological molecules using techniques like X-ray diffraction, there has been very little progress in obtaining information on the time-dependent conformational changes that are the key to understanding the function of such molecules. The importance of extensive motion of protein domains coupled to biological electron transfer has come to light in recent years, primarily through structural analysis of redox proteins using crystallographic methods [1,2]. Domain motion shortens electron tunneling distances through conformational search mechanisms, thus increasing the probability of electron transfer by quantum-mechanical tunneling [3]. Despite these advances, real-time observation of conformational change coupled to electron transfer is difficult and we lack a method of directly probing conformational change linked to redox chemistry in real time that is generally applicable to biological macromolecules. In this work we show that the introduction of surface-exposed cysteine groups into proteins can lead to the creation of protein-Au(110) assemblies suitable for combined electrochemical and reflectance anisotropy spectroscopy (RAS) studies of conformational changes driven by electron transfer. We report real-time measurements of changes in RA spectra that are correlated with the changes in electrode potential. In natural systems such changes in electrode potential induce electron transfer interactions that lead to changes in the conformation of protein domains.

The experiments were performed on the electron transfer flavoproteins (ETFs) that act as carriers of electrons between a number of donor and acceptor proteins [4]. They are dynamic molecules both in complex with donor proteins [1,2] and free in solution [5,6]. They have a 2-electron reduction capacity, but thermodynamically are restricted to 1-electron reduction/oxidation in biological cells. The crystallographic structure of human and bacterial ETFs are known [1,7], thus facilitating a knowledge-based design of surface-exposed cysteine groups.

ETF is made up of two relatively rigid units, the first made up of domains I and III and the second of domain II, joined together by a small “hinge” region (fig. 1). It was modified using standard mutagenesis procedures [8] to introduce a surface-exposed cysteine residue by replacing residue Asp-16 by Cys-16 (D16C ETF) or Ser-193 by Cys-193 (S193C ETF) using synthetic DNA oligonucleotides. Expression and purification of the mutant ETF enzymes were by published procedures [9], except that mercaptoethanol (2 mM) was added to all buffers during the purification and the storage of the engineered proteins in order to keep the thiol in reduced form prior to immobilisation on an Au(110) surface.

Prior to the RAS experiments the mid-point reduction potential of the FAD cofactor in the engineered ETF proteins was determined as described previously for a wild-type ETF [9]. Enzyme solutions were electrochemically titrated using sodium dithionite as reductant and potassium ferricyanide as oxidant. The UV-visible spectra of ~30 to 40 points from 300 to 800 nm (4.1 to 1.6 eV) across a whole range of redox potentials during the process of reduction and oxidation were recorded and no hysteretic behaviour was observed. Plots of the absorbance values of 2.6 eV (470 nm) and 3.3 eV (370 nm) against the redox potential (fig. 2) were fitted using eq. (1), which represents a 2-electron redox process derived by the extension of the Nernst equation and the Beer-Lambert law [9,10]:

$$A = \frac{a10^{(E-E'_1)/59} + b + c10^{(E'_2-E)/59}}{1 + 10^{(E-E'_1)/59} + 10^{(E'_2-E)/59}}. \quad (1)$$

A is the absorbance value (at either 470 nm or at 370 nm) at the electrode potential E , and a , b and c are the component absorbance values of the fully oxidized (ETF_{ox}), single-electron reduced ($\text{ETF}_{1\text{e}}$) and fully reduced states ($\text{ETF}_{2\text{e}}$), respectively. E'_1 and E'_2 are the midpoint potential for oxidized/ $\text{ETF}_{1\text{e}}$ and $\text{ETF}_{1\text{e}}$ / $\text{ETF}_{2\text{e}}$ couples, respectively. As with wild-type ETF [9,11], it was not possible to reduce the engineered proteins to the 2-electron reduced species using dithionite; reduction to the 1-electron level was readily achieved. The measured redox potentials relative to the standard hydrogen electrode (SHE) for the $\text{ETF}_{\text{ox}}/\text{ETF}_{1\text{e}}$ couple of the S193C ($E'_1 = +64 \pm 2 \text{ mV}$) and D16C ($E'_1 = +50 \pm 2 \text{ mV}$) proteins are ~90 mV less than for the wild-type ETF (+150 mV). This might reflect conformational differences between wild-type and mutant proteins that affect the local environment around the FAD isoalloxazine ring [12]. As with the wild-type ETF, the $\text{ETF}_{1\text{e}}/\text{ETF}_{2\text{e}}$ couple was $\ll -250 \text{ mV}$ for the engineered ETF proteins and not accessible [9] using this method.

RAS is a surface-sensitive optical technique that has been used to probe molecular assembly and changes in molecular orientation at metal/liquid interfaces [13-17]. The preparation of the Au(110) single crystal used to immobilize engineered variants of ETF together with a description of the electrochemical cell, potentiostat and RAS instrument [18] have been described previously [15]. Electrolyte solutions were prepared from 0.1 M NaH_2PO_4 and K_2HPO_4 dissolved in ultra-pure water. The RAS signal is given by

$$\{\text{Re}\} \left\{ \frac{\Delta r}{r} \right\} = \{\text{Re}\} 2 \left\{ \frac{r_{[110]}^- - r_{[001]}}{r} \right\}. \quad (2)$$

RAS is extremely sensitive to the electronic structure and the morphology of both the Au(110) surface and the adsorbed molecules [13,16,17,19,20]. The RA spectra of the bare Au(110) surface as the potential was varied from +300 to -356 mV vs. natural hydrogen electrode (NHE) were found to vary in a similar manner to those observed previously in this electrolyte [14]. In separate experiments solutions of each engineered ETF protein contained in 50 mM potassium phosphate buffer, pH 7.0, were loaded at +244 mV vs. NHE in the electrochemical cell to give a final concentration of 2 μM . Spectra were collected for the

same range of potentials as collected for the Au. The addition of the proteins gave rise to changes in the Au spectra as a function of the potential, some of them are characteristic of the formation of a Au-thiolate bond [14] (fig. 3(a)) indicating that the proteins bonded to the surface through the cysteine thiols on the surface of the protein in the α -subunit of the α/β heterodimer (fig. 1) thus anchoring the larger of the two units, domains I and III, to the Au surface leaving the upper unit, domain II, to move freely about the “hinge”. Compared to the RA spectrum of the clean Au(110) surface, the protein adsorption shifted the negative peak at ~ 2.6 eV to 2.5 eV and reduced its intensity. Protein adsorption also decreases significantly the intensity of the 3.6 eV negative feature in the RAS of the clean Au(110) surface. The RAS intensity at 2.5 eV is a sensitive monitor of the changes induced in the spectrum by the addition of the proteins and by the variation of the potential applied to both the clean Au(110) surface and the surface after adsorption of the proteins. In separate experiments we found that changing the applied potential on the bare Au(110) electrode successively from negative values through 0 to positive values resulted in an increase in the intensity of RA signal at 2.5 eV but when the protein is adsorbed onto the Au(110) there is a larger decrease in the RA signal at 2.5 eV on following the same voltage changes. Figure 3(b) shows the RAS of the S193C/Au(110) assembly at potentials of +300mV, -100 mV and -356 mV vs. NHE after subtracting the RAS of the the Au(110) at the corresponding potentials. Similar results are obtained for the D16C/Au(110) assembly. These changes were found to be reversible on time scales of the order of 100 seconds.

The calculation of the RA spectra of adsorbed molecules on surfaces is difficult and requires a good knowledge of the geometry of the adsorption site and the local electronic structure. Consequently, it is not possible to interpret the spectral features of the RAS from first principles and we concentrate on the interpretation of the changes in the spectra induced by varying the applied potential. We have established that the redox potentials of S193C and D16C are $+64 \pm 2$ mV and $+50 \pm 2$ mV, respectively, and that the second redox potentials are $\ll -250$ mV for both forms. We argue that the conformational changes associated with changes in the charge states of these molecules give rise to changes in the RAS of the molecules adsorbed at the Au(110)/electrolyte interface and that the RAS observed at 2.5 eV is a sensitive monitor of these changes. Alternative explanations of the results are discussed below. The solid line in fig. 4(a) shows the intensity of the RAS at 2.5 eV as the adsorbed S193C protein was cycled between +300mV and -100 mV. This gives rise to a reversible 1-electron oxidation/reduction cycle between the oxidised and the 1-electron reduced species and the associated conformational changes are revealed by the RAS. Figure 4(a) also shows ($\times$ and $\circ$) that RAS is also able to monitor the change resulting from cycling the potential to -356 mV which will drive the protein to the 2-electron reduced form which can only be produced electrochemically [21]. Similar results are found for D16C. Finally, fig. 4(b) shows the results of stepping the potential applied to the D16C/Au(110) and S193C/Au(110) assemblies in the sequence +300mV, -100 mV, -356 mV, -100 mV, +300mV which successively takes the proteins stepwise from i) the oxidised state to the 1-electron reduced, ii) the 2-electron reduced to the 1-electron reduced and iii) from the 1-electron reduced back to the oxidised state. The changes reported in fig. 4(b) are not completely reversible and show a slight drift. This drift could arise from the loss of some ETFs from the electrode to the solution during recycling and/or the loss of FAD from the molecule, thus preventing further change in conformation.

There are a number of possible explanations for the spectral changes reported in figs. 2 to 4. RAS is a local probe of dipole transitions on molecular length scales and the intensity of a dipole transition observed in RAS is determined by the geometrical relationship between the dipole transition and the electric-field vector (E) of the incoming polarised light. Since the orientation of the dipole transition is fixed with respect to the molecular axes, the RAS signal monitors the relationship between the orientation of the molecule in space and the

fixed direction of the E vector. Given the structure of this molecule, such transitions will be located in small regions of either the upper or lower unit.

The changes that we observe could be due to intrinsic changes in the electronic structure of the FAD cofactor that allowed the population of the electron energy levels in molecular regions of the protein to change. This would allow a redistribution of charge that could quench or alter the orientation of the dipole transitions. However, if the electronic structure of the protein was such as to allow this to happen, then it would happen in the natural species and the change in orientation as a result of electron transfer that is necessary for the protein to carry out its function would not occur since the protein would be able to “soak up” electrons rather than transfer them. The more likely explanation is that the changes in the RAS that we observe arise from the conformational changes that accompany electron transfer in the natural protein.

Alternatively, our results could be associated with changes in the interactions between the oxidised and reduced FAD cofactor forms and the protein cavity in which they reside and it is likely that there will be some local change in the environment around the FAD isoalloxazine ring, the part that accepts the electron. However, this is an unlikely explanation of our results since the FAD remains essentially bound in a similar, but perhaps not identical, conformation in both redox states. It is the surrounding residues that move. The Arg-237 stacks over the FAD and has multiple interacting H-bonding partners that enable ETF to explore a number of different conformational states in solution. The distribution of conformational states is influenced by the redox state of the FAD isoalloxazine ring [12].

Another possible explanation for the results is that they are associated with changes in the solvent accessibility to the FAD-containing protein cavity upon reduction/oxidation and, as the FAD binding domain rocks about the isoalloxazine ring, the FAD will become more solvent accessible for different extremes of the motion [5,13]. Models of such motion have been developed on the basis of crystal structures for wild-type and mutant ETFs [12] and small-angle X-ray scattering studies of ETF [5]. If the dipole transition that contributes to the RAS of the protein is located on a part of the protein cavity that is fixed, then variations in its exposure to the solvent could in principle change the optical response. However, since variations in solvent exposure are dependent on the domain motion that we are seeking to monitor, then this would simply mean that the changes in the RAS are a consequence rather than a direct monitor of the domain motion.

Our preferred explanation for the results is that they arise from changes in the orientation of the upper unit, domain II, of the adsorbed proteins on the electrode and are associated with the conformational changes that are induced in the proteins by electron transfer and that are the basis of their biological function. In support of this view we note that there is a significant region of unfilled space for the upper unit to “explore” by varying its orientation with respect to the lower unit through motion about the “hinge” and that small-angle X-ray scattering data on crystals of ETF in complexes are consistent with the movement of the flavin binding domain [5]. The functional behaviour of the protein depends upon the transfer of electrons to adjacent protein units and this requires the upper unit to change its orientation with respect to the lower unit in response to the passage of an electron from the lower unit to the upper one. We observe a change in RAS signal resulting from the transfer of an electron that is known to induce a relative motion of the two units when the protein carries out its function. Furthermore, the changes in the RAS signal happen at the specific changes in voltage that are known to induce the transfer of an electron between the two sub-units of the protein.

In conclusion we provide evidence that a combination of the electrochemical reduction of engineered ETF proteins with RAS can probe directly conformational events linked to electron transfer in biological macromolecules. Our study demonstrates a general strategy for studying directly conformational events linked to electron transfer. This approach should open up a detailed analysis of the kinetics of interchange between, and equilibrium distributions of, different protein conformers triggered by redox processes in biology.

Acknowledgments

The authors gratefully acknowledge funding from the UK Biotechnology and Biological Sciences Research Council and the UK Engineering and Physical Sciences Research Council. NSS is a BBSRC Professorial Fellow.

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Fig. 1. Molecular graphics ribbon diagram for the structure of *M. methyltrophus* ETF showing the mutation sites where a cysteine residue was introduced by directed mutagenesis, replacing either the aspartate-16 or the serine-193 residues on the surface of the protein to produce, respectively, the D16C and S193C mutants. The sites of modifications are shown as spheres. The α -subunit of the enzyme is shown in dark grey, the β -subunit in light grey and the FAD in black.

Fig. 2. Spectral changes accompanying titration of the engineered S193C ETF during potentiometric measurements. Black arrows indicate the direction of absorbance changes at 2.6 eV (470 nm) and at 3.3 eV (370 nm) as the potential is reduced. Inset: plot of absorbance change at 470 nm (•) and at 370 nm (○) against the recorded potentials for the engineered S193C ETF protein. $E'_1 = +64\text{mV}$, $E'_2 \ll -250\text{mV}$. The ETF_{2e} is inaccessible on reducing the engineered protein chemically as observed for a wild-type protein [9]. However, the reduction of ETF to the 2-electron reduced species is achievable by electrochemical methods [19]. Protein ($63\text{ }\mu\text{M}$) was contained in 50 mM potassium phosphate buffer, pH 7.0 at $25\text{ }^\circ\text{C}$ and maintained under anaerobic conditions.

Fig. 3. RA spectra of the engineered ETF protein assembled on the Au(110) electrode surface in 0.1 M $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$. (a) RA spectrum of the Au(110) electrode (solid line); RA spectrum of the Au(110) electrode assembled with the engineered S193C ETF protein (dashed line); RA spectrum of the S193C ETF after subtracting the Au(110) spectrum ($\circ$), all obtained at +244mV, vs. NHE. (b) RA spectra of the engineered S193C ETF protein assembled on the Au(110) electrode surface at +300mV (solid line), -100mV (dashed line) and at -356mV ($\circ$) representing the RA spectra of the oxidised, one-electron reduced and two-electron reduced species of the S193C ETF protein, respectively. In each case the RA spectrum of the Au(110) obtained at the corresponding potential has been subtracted from that of the adsorbed protein.

Fig. 4. Time dependence of the spectral intensity of the RAS signal at 2.5 eV following a stepped voltage change recorded for the engineered ETF proteins adsorbing onto the Au(110) electrode. Proteins were contained in 0.1 M $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$. (a) For the S193C ETF protein, the voltage is stepped from +300mV to -100mV (solid line), from +300mV to -356mV (x) and from -356mV to -100mV (o) for 10 cycles each of 60 s. The spectra are offset for clarity and the scale, 1×10^{-3} , is marked on the y-axis. (b) The voltage is stepped from +300mV to -100mV to -356mV and then back to -100mV and finally to +300mV. The trace for the S193C ETF protein is the solid line and for the D16C ETF protein is shown by circles with each voltage maintained for 60 s. The spectra are offset for clarity and the scale, 1×10^{-3} , is marked on the y-axis.