The influence of the structure of the Au(110) surface on the ordering of a monolayer of cytochrome P450 reductase at the Au(110)/phosphate buffer interface

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The reflection anisotropy spectra (RAS) observed initially from Au(110)/phosphate buffer interfaces at applied potentials of –0.652 and 0.056 V are very similar to the spectra observed from ordered Au(110) (1 × 3) and anion induced (1 × 1) surface structures respectively. These RAS profiles transform to a common profile after cycling the potential between these two values over 72 h indicating the formation of a less ordered surface. The RAS of a monolayer of a P499C variant of the human flavoprotein cytochrome P450 reductase adsorbed at 0.056 V at an ordered Au(110)/phosphate buffer interface is shown to arise from an ordered layer in which the optical dipole transitions are in a plane that is orientated roughly normal to the surface and parallel to either the [110] or [001] axes of the Au(110) surface. The same result was found previously for adsorption of P499C on an ordered interface at –0.652 V. The adsorption of P499C at the disordered surface does not result in the formation of an ordered monolayer confirming that the molecular ordering is strongly influenced by both the local structure and the long range macroscopic order of the Au(110) surface.

1 Introduction

While there has been considerable progress in determining the structure of proteins using techniques like X-Ray diffraction, there has been much less progress in measuring the conformational changes that are the key to their function. This lack of progress in monitoring dynamical changes in proteins is due to the dearth of experimental techniques for monitoring changes in molecular structure in real time. Recently the technique of reflection anisotropy spectroscopy (RAS) has been used to monitor changes in the orientation of molecules adsorbed at Au(110)/liquid interfaces and has been shown to have the potential to monitor conformational changes in surface adsorbed proteins on a millisecond timescale. This paper is part of a long term program [1–3] directed at understanding the interaction between human flavoprotein cytochrome P450 reductase (CPR) [4–6] and Au(110)/phosphate buffer interfaces with the aim of using RAS to monitor conformational change in this electron transfer flavoprotein. Cytochrome P450 reductase is chosen for these studies because it is an electron transfer flavoprotein that in living systems is anchored to a membrane and which carries out its electron transfer function by large changes in the relative orientation of two structural parts of the protein: the FAD and FMN binding domains. It is thus possible in principle to induce conformational change in the protein by varying the potential applied to the Au(110) electrode and monitoring the change in real time using RAS. However prior to such studies it is necessary to establish the symmetry of cytochrome P450 reductase adsorbed at Au(110)/phosphate buffer interfaces at different potentials. This precondition is completed in the research reported in this paper.

An earlier paper [1] studied a variant CPR P499C, in which the 499th amino acid in the protein sequence, proline, was replaced by a cysteine residue. This change resulted in the molecule attaching to the Au(110) surface by a Au–S bond (Fig. 1). The previous work [1] established the
normal to the surface and parallel to either the adsorption at phosphate buffer interface. It is also shown that, as found for give rise to an ordered structure at a less ordered Au(110)/phosphate buffer interfaces. The earlier work studied ordered monolayers and bilayers adsorbed at ordered Au(110)/phosphate buffer interfaces. The earlier work studied ordered monolayers of P499C adsorbed on ordered Au(110) surfaces at an applied potential of $-0.652 \text{ V}$, a potential corresponding to P499C in its most reduced state. In this work it is shown that adsorption from a solution that gave rise to an ordered monolayer in the previous work does not give rise to an ordered structure at a less ordered Au(110)/phosphate buffer interface. It is also shown that, as found for adsorption at $-0.652 \text{ V}$, the adsorption of a monolayer of P499C on an ordered Au(110) surface at a potential of 0.056 V, which corresponds to the oxidised state, also forms an ordered structure in which the optical transitions giving rise to the RAS profile lie in a plane that is orientated roughly normal to the surface and parallel to either the [110] or [001] axes of the Au(110) surface.

2 Experimental As in previous work [1–3, 7, 8] experiments were performed on a Au(110) single crystal of 99.999% purity in the form of a disc of diameter 10 and 2 mm thick with an exposed surface area of 0.5 cm². The crystal was orientated to an accuracy of 0.1° by X-ray diffraction and prepared for each experiment by mechanically polishing on successively smaller grades of diamond paste down to 0.25 μm followed by flame annealing with a butane micro torch and protected with a drop of ultrapure water before being transferred into an electrochemical cell. The electrochemical cell and potentiostat have been described previously [1, 7, 8]. All potentials are referenced to a saturated calomel electrode (SCE). The experiments employed a reflection anisotropy spectrometer (RAS) that has also been described previously [1, 7–10]. This yields a linear optical signal that is the difference in reflectivity from two orthogonal directions in the surface and plane polarised light at normal incidence and reflection from the crystal. For a cubic substrate this geometry results in a cancellation of the bulk signal by symmetry and RAS becomes a probe of surface anisotropy [7]. The measured RA signal from 1.5 to 5.5 eV is given by

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

where $r_{[110]}$ and $r_{[001]}$ are the reflection coefficients in the [110] and [001] directions in the (110) surface respectively. The sum of these quantities is given by $r$ and the average by $r/2$. Azimuthal dependent RA spectra were measured as a function of the azimuthal angle around the direction of the normal incidence light.

As in the previous work [1, 2, 7, 8] the solutions used were NaH2PO4, K2HPO4, (BDH, Analar grade) prepared with Millipore ultrapure water (18 MΩ cm) and made oxygen free by purging with argon prior to use. P499C was added to the electrochemical cell. The potentials of the oxidised and reduced forms of P499C have been determined [1].

3 Results The RAS profile of the initial Au(110)/phosphate buffer interface obtained at applied potentials of $-0.652$ and 0.056 V corresponding to the potential which gives rise to the most reduced and the oxidised forms of the protein respectively are shown in Fig. 2a. Figure 2b shows the change in the RAS profile of the Au(110) electrode held at 0.056 V when a solution of P499C is added to the electrochemical cell at a concentration and pH that is known to give rise to an adsorbed monolayer of the protein [1]. The difference between the two RAS profiles obtained at 0.056 V is also shown in Fig. 2b.

The results shown in Fig. 2a confirm that the RAS profile of the Au(110) surface in an electrolyte is very sensitive to the applied potential, changes in which give rise to changes in surface morphology [10–16] similar to those associated with the RAS profiles of different morphological structures of the Au(110) surface in ultra high vacuum (UHV) [10, 17]. The relationship between the RAS profile and the structure of the Au(110) surface has been discussed extensively in previous work [10–17]. The black line in Fig. 3 shows the RAS signal obtained at 2.7 eV as a function of time when the potential applied initially to the Au(110) electrode is held at $-0.652 \text{ V}$ for 300 s and then switched to 0.056 V. This results in an initially rapid change in intensity which is completed more slowly over ~60 s to the intensity measured at 0.056 V in Fig. 2a. Returning the potential to $-0.652 \text{ V}$ after another 300 s results in a more rapid transition, completed in <5 s, to the signal associated with the initial profile obtained at $-0.652 \text{ V}$. However as this switching process is continued over long time periods the RAS profiles
obtained at the two potentials converge. This is shown by the grey line in Fig. 3 and the RAS profiles in Fig. 4 which were obtained after this potential stepping procedure had been carried out for 72 h. Figure 5 shows the RAS profile of the Au(110)/electrolyte interface produced by stepping between the two potentials for 72 h and held at $-0.652$ V together with the RAS profile obtained following the addition of a solution of P499C with the concentration and pH that in earlier work [1] was shown to lead to the adsorption of a monolayer of the protein. Only very slight changes in the spectral profile, notably above $\sim 5.2$ eV, indicate that the P499C has adsorbed at the interface.

The conditions under which monolayers of P499C adsorb at the Au(110)/electrolyte interface depend quite sensitively on the pH and concentration of the solution [1]. Previous work has shown that considerable insight into the orientation of adsorbed molecules on surfaces can be obtained by collecting RA spectra as a function of the azimuthal angle around the direction of the normal incident light beam [1, 7, 8, 10]. The RAS profile of a monolayer of P499C adsorbed at the Au(110)/phosphate buffer interface at an applied potential of $0.056$ V, corresponding to the oxidised form of the protein, is shown in Fig. 6 as a function of time.

Figure 2 (a) RA spectra of Au(110) at $-0.652$ V (black line) and $0.056$ V (grey line) before stepping the potential applied to the Au (110) surface. The line shows the energy at which the RAS signal was monitored as the applied potential was switched (Fig. 3). (b) RA spectra of Au(110) (grey line) and of a monolayer of P499C adsorbed on Au(110) ($\bigtriangleup$) at $0.056$ V. The subtraction of these spectra is shown by (○). All spectra recorded in 0.1 M NaH$_2$PO$_4$/K$_2$HPO$_4$ at pH 7.2. It is important to note that the RAS profiles shown in (a) correspond to the same zero on the vertical scale since they were obtained in subsequent experiments. The same is true for the two profiles shown in (b).

Figure 3 Change in RA intensity as a function of time recorded at 2.7 eV while the potential is switched from $-0.652$ to $+0.056$ to $-0.652$ V every 300 s, first run (black line) and the last run after 72 h (grey) are plotted on the primary y-axis with the corresponding reflectivity on the secondary y-axis on the right of the figure. All the runs are recorded in 0.1 M NaH$_2$PO$_4$/K$_2$HPO$_4$ at pH 7.2.

Figure 4 RA spectra of Au(110) at $-0.652$ V (black line) and at $0.056$ V (grey line) after stepping between $-0.652$ and $0.056$ V for 72 h recorded in 0.1 M NaH$_2$PO$_4$/K$_2$HPO$_4$ at pH 7.2.
of the azimuthal angle around the direction of the normal incident light beam. It is notable that the RAS profiles shown in Fig. 6 is essentially flat across the spectral range when measured along the principal axis of the Au(110) surface, $\theta = 90^\circ$.

4 Discussion

The RAS of Au(110)/electrolyte interfaces is very sensitive to the potential applied to the Au(110) electrode and RAS profiles corresponding to the $(1 \times 1)$ surface structure and the $(1 \times 2)$ and $(1 \times 3)$ reconstructions and an anion induced reconstruction of the Au(110) electrode in a variety of electrolytes have been identified [11, 12]. The RAS profile observed in the phosphate buffer at $-0.652$ V is similar to that observed from a Au(110) $(1 \times 3)$ surface reconstruction and that observed at $0.056$ V is similar to that observed from an anion induced structure observed at Au(110)/electrolyte interfaces in a variety of electrolytes [11, 12]. RAS is a local probe that depends on macroscopic anisotropy. The intensity of a feature in the RA spectrum is a product of the intrinsic strength of the feature, the number of sites and the degree of anisotropy of the surface [10, 18]. The sensitivity of the RAS profiles to the morphology of Au(110) surfaces is due to the dependence of the signal strength on both the local atomic structure and the macroscopic anisotropy of the surface. It is clear from previous work [11, 12] that both these factors are strongly influenced by the flame annealing process and, in electrolytes, by the potential applied to the Au(110) electrode. Recent results [12] obtained on the time dependence of changes observed in the RAS profile of the $(1 \times 2)$ reconstruction of the Au(110)/electrolyte interface and its sensitivity to the stability of steps, surface states and the adsorption of anions lead us to suggest that the stepping of the potential applied to the Au(110)/electrolyte surface between $-0.652$ and $0.056$ V over 72 h results in the formation of a disordered and possibly mixed Au(110)/phosphate buffer surface structure. This view is supported by the fact that the RAS profiles obtained at the end of the potential stepping procedure cannot be reproduced by any combination of the RAS signatures identified for the $(1 \times 1)$, $(1 \times 2)$, $(1 \times 3)$ and anion induced surface structures. This finding together with the convergence of the RAS profiles of the Au(110)/phosphate buffer interface obtained at $-0.652$ and $0.056$ V following the cycling of the potential between these two values (Figs. 2 and 3) indicates that the initial $(1 \times 3)$ and anion induced surface structures present at these potentials are destroyed by the stepping procedure. However the strong RAS signal shown in Fig. 5 demonstrates that the surface still shows significant macroscopic anisotropy. This anisotropy is probably due to the formation of a large number small domains with a variety of surface structures. The underlying symmetry of the Au(110) surface will impose a macroscopic order on the orientations of these domains, which gives rise to RAS profile, but there will be no long range order. The difference between the two profiles in the region 3.3–3.7 eV is a weak representation of a characteristic feature of the profile of the anion induced surface structure [11, 12]. This indicates that when the potential is changed to 0.056 V anions adsorb on the disordered surface in a similar way to their adsorption on an ordered surface.

In earlier work we established that, as expected from experiments with other S-containing compounds [1, 2, 19, 20], the P499C adsorbed on the ordered Au(110)/phosphate buffer interface at $-0.652$ V through the formation of a Au–S bond identified by a characteristic feature in the RAS at $\sim 2.54$ eV, which appears to low energy of the feature at 2.6 eV in the RAS profile of Au(110). This feature is also present, though significantly weaker and shifted to 2.6 eV by overlap with the gold spectrum, in the RAS profile of P499C adsorbed on the ordered anion induced Au(110) surface at $0.056$ V (Fig. 2b). Careful measurements of the variation in the intensity of this feature as a function of changes in the applied potential show that it has the behaviour expected of the feature associated with the Au–S bond [1, 3, 19, 20] and leads us to conclude that the adsorption of P499C onto the Au(110) surface at $0.056$ V also occurs through the formation of a Au–S bond.

The slight differences in the RAS profiles shown in Fig. 4 indicate that P499C adsorbs onto this surface at $-0.652$ V but the lack of any strong differences in the profiles and in particular the absence of the signature of the Au–S bond, which is more intense at $-0.652$ than at $0.056$ V [3, 19, 20], indicates that the molecules adopt a macroscopically disordered structure with no significant net anisotropy. The lack of macroscopic anisotropy observed from P499C adsorbed on the macroscopically anisotropic Au(110) surface produced by stepping the applied potential probably arises from the mechanism by which an ordered molecular monolayer is formed on the Au(110) surface. The earlier study of the kinetic behaviour of the RAS profile [1] indicated that this was a two-stage process. An initial saturation of the surface by molecules adsorbed through the formation of Au–S bonds was followed by a much slower ordering of the randomly adsorbed species that increased the anisotropy. This ordering process resulted in a monolayer in

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**Figure 6** RA spectra of a monolayer of P499C CPR adsorbed on Au(110) at 0.056 V versus SCE at 45° (grey line), 55° (△), 65° (○), 75° (●), 85° (+), 90° (×), 95° (□), 105° (◇), 115° (●), 125° (△) and 135° (black line) all recorded in 0.1 M NaH$_2$PO$_4$/K$_2$HPO$_4$ at pH 7.2.
which the optical axes of the molecules were orientated along either the [110] or [001] directions of the Au(110) (1 x 3) surface and is clearly driven by the underlying long range order of the substrate. The lack of any long range order on the Au(110) surface produced by cycling the applied potential means that there is no mechanism to facilitate the ordering of the randomly adsorbed molecules.

The RAS profile of P499C adsorbed on the ordered Au(110)/phosphate buffer interface at 0.056 V shows the characteristic feature associated with the formation of a Au–S bond (Figs. 2 and 5). The differences between the RA spectra of Fig. 2b are similar to those observed previously from ordered monolayers of P499C adsorbed on Au(110) surfaces [1]. Apart from a broad contribution centred on ~2.0 eV these differences arise from the spectra of the isoalloxazine rings. In particular the Au–S feature merges into and overlaps with one of the transitions associated with the isoalloxazine rings [1]. It is also found that the RAS of the protein adsorbed on the ordered surface at 0.056 V (Figs. 2 and 5) is obtained by adsorbing the protein at ~0.652 V and changing the applied potential to 0.056 V. This last result indicates that the macroscopic arrangement of the protein on the ordered Au(110) surface is essentially the same when the protein is adsorbed at either of these applied potentials. In solution the spectrum of an isoalloxazine has three main contributions, a peak at 2.7 eV which is ~1.0 eV wide, a band between 3.3 and 3.8 eV and a stronger band peaking at 4.6 eV [21–23]. The contribution of these three transitions to the RAS profile of the adsorbed protein will depend on their orientations with respect to the Au(110) surface. These three transitions are primarily located in the plane of the isoalloxazine ring and polarised, in different directions, but largely along the long axis of the ring [21–23]. This is a similar situation to that analysed in detail for cytosine [7] and adenine [8] adsorbed at Au(110)/phosphate buffer interfaces where it was shown that if the RAS profile is essentially flat across the spectral range when measured along the principal axes of the Au(110) surface then the plane of the molecules must be oriented roughly vertical to the surface and parallel to one of the principal axes directions of the Au(110) surface [7]. This result follows from the fact that the contribution of a dipole transition to the azimuthal RAS profile of the adsorbed molecule will have a cos2θ dependence where θ is the projection of the direction of the dipole moment on to the Au(110) surface. If, as found in Fig. 6, the whole spectral profile has a cos2θ dependence and goes to zero along the principal axes of the Au(110) surface then all the dipole transitions must be in a plane vertical to the surface and aligned along one of the principle axes [7]. It is difficult to determine the precise orientation of the planes of the isoalloxazine rings relative to the Au(110) surface since along the principal axes of the Au(110) the RAS profile is very sensitive to the azimuthal angle and the accuracy with which this angle can be determined in the Au(110) surface is ±4°. This translates into a tilt angle sensitivity of the plane of at worst ±10° which occurs when two transitions in the plane are 50° apart and lie in directions ±25° from a principal axis of the Au(110) [7]. Although theoretical results do not agree on the directions of the dipole transitions with respect to the axes of the isalloxazine ring they do not predict large differences in direction, one calculation putting the maximum divergence between two of the directions of the dipoles at 25° [22] and another at 11° [23]. The azimuthal data of Fig. 6 indicates that the direction of the isoalloxazine ring planes is along one the principal axes of the Au(110) to an accuracy of ±4° and the results of Fig. 6 are consistent with all contributions to the RAS profile showing a cos2θ dependence indicating that there are no significant dipole contributions with a projection onto the Au(110) surface other than those along the axis of the planes.

5 Conclusions It has been demonstrated that the characteristic RAS profiles observed initially at applied potentials of ~0.652 and 0.056 V from the Au(110)/phosphate buffer interface prepared by flame annealing transform to a common profile after cycling the potential between these two values over 72h. This indicates that the initial differences in the local structure and macroscopic order of the Au(110) electrode held at these two potentials are lost as a result of the potential cycling. The absence of significant differences between the RAS profile of the Au(110)/phosphate buffer interface created by the potential cycling and the RAS profile following adsorption of P499C at this interface confirms that the formation of an ordered monolayer on the Au(110) surface at ~0.652 V is strongly influenced by both the local structure and the long range macroscopic order of the Au(110) surface.

The azimuthal dependence of the RAS profile of a monolayer of P499C adsorbed on a well ordered Au(110)/phosphate buffer interface at 0.056 V establishes that the optical dipole transitions that give rise to the spectra must be lie in a plane orientated roughly normal to the surface and directed along either the [110] or [001] axes of the Au(110) surface.

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