Immobilization of the Glucose-Galactose Receptor Protein onto a Au Electrode Through a Genetically Engineered Cysteine Residue

Jianbin Wang, a Linda A. Luck, b,c and Ian I. Suni a,b,d

*Department of Chemical and Biomolecular Engineering, and bDepartment of Chemistry and Biomolecular Science, Center for Advanced Materials Processing, Clarkson University, Potsdam, New York 13699-5705, USA

Manuscript submitted August 9, 2006; revised manuscript received October 5, 2006. Available electronically December 22, 2006.

Members of the bacterial periplasmic binding protein (bPBP) superfamily have been studied for biosensor applications due to their solubility, stability, and the large conformational change they undergo upon ligand binding.1-3 Glucose biosensors have been demonstrated by immobilizing the glucose-galactose receptor (GGR) protein, a member of the bPBP superfamily, onto flat Au electrodes through several detection strategies, including quartz crystal microbalance (QCM),4 surface plasmon resonance (SPR),5,6 and electrochemical impedance spectroscopy (EIS).7

The GGR protein has no native cysteine residues and thus no disulfide bonds. This makes it an ideal candidate for immobilization onto Au electrodes by introducing cysteine residues by genetic engineering, allowing formation of covalent Au-S bonds. This holds the potential for oriented protein immobilization, which is highly desirable for the development of biosensors, artificial organs, and other biotechnologies.8 Au is commonly used as the electrode material in electrochemical biosensors, given its greater biocompatibility than other metals. Here, immobilization onto a Au electrode of GGR A1C, where alanine at the N-terminus is replaced by cysteine, is studied.

We recently demonstrated an impedance biosensor for the GGR A1C immobilized onto a Au electrode.7 Here, the nature of protein immobilization in this system is studied further, including an approximate determination of the dissociation constant (Kd) for the Au-immobilized GGR A1C protein. Although several research groups have immobilized proteins through cysteine groups onto flat Au electrodes, the detailed nature of this interaction has not been studied.4,7,10,17

Experimental

Expression and purification of GGR A1C and wild-type GGR.—The wild-type GGR and GGR A1C plasmids were expressed in E. coli BL21 cells and purified as described previously.18 Protein was then dialyzed against two exchanges of 3 M guanidine chloride (GnHCl), 100 mM KCl, 20 mM EDTA, 10 mM tris pH 7.1, and four exchanges of refolding buffer containing 100 mM KCl, 10 mM tris pH 7.1, and 0.5 mM CaCl2.

Electrode preparation.—Glass slides with a 100 nm Au film atop a 5 nm Ti adhesion layer were purchased from Evaporated Metal Film Corporation (Ithaca, NY). The electrode was fixed by an O-ring into an electrochemical cell constructed from virgin Teflon with an electrode area of 0.28 cm² and a cell volume of 5 mL. The electrode was cleaned electrochemically in 0.1 M H2SO4 and 0.01 M KCl by potential scanning ten cycles between 0.16 and 0.71 V, three cycles between 0.16 and 1.01 V, and three cycles between 0.16 and 1.46 V vs saturated calomel electrode (SCE).19

The Au electrode was exposed to 50 μL of solution containing 27 μM GGR protein; then, the cell was incubated for 3–5 h. The Au electrodes were subsequently thoroughly rinsed in 10 mM pH 7.1 tris buffer containing 0.1 M KCl and 0.5 mM CaCl2, and then immersed into the buffer solution.

Instruments.—Impedance measurements were obtained with a standard three-electrode electrochemical cell, using a Pt counter electrode and an SCE reference electrode. Impedance spectra were obtained using a Bioanalytical Systems (BAS) model 100B/W electrochemical workstation, with a built-in multisine ac impedance module. The impedance response took about 4 min to obtain from 1.2 to 950 Hz, at 10 steps per decade. Impedance measurements at a single frequency were made with a Princeton Applied Research (PAR) model 263A potentiostat and a Stanford Research Systems SR830 lock-in amplifier for phase-sensitive detection.

Experimental methods.—The electrode was first immersed into the test solution, pH 7.1 10 mM tris buffer, 0.1 M KCl, and 0.5 mM CaCl2, with or without Fe(CN)6 3−/4−. The open-circuit potential (OCP), where the net current is zero, was measured, and the dc potential during subsequent measurements was set to this value. In solutions without a redox probe, the OCP varies from 85–95 mV vs SCE, while in solutions containing 0.5 mM Fe(CN)6 3− + 0.5 mM Fe(CN)6 4−, the OCP is approximately 186 mV vs SCE. After measurement of the impedance of the biosensor interface, glucose in the same refolding buffer was added to the cell to the desired final concentration. To test for steady state, the impedance of the biosensor interface was measured every 15 min until no change was observed in successive measurements. The Nyquist plots were fit to an equivalent circuit by complex nonlinear least-squares (CNLS) methods using Z-View, from Scribner and Associates.

Results and Discussion

Initial studies of GGR A1C protein immobilization used linker molecules to attempt to form an oriented protein film on Au by indirect methods. A sulphydryl-terminated self-assembled monolayer (SAM) was formed on a Au surface,20 and the GGR A1C protein was chemically bound through formation of a disulfide bond. In this case the interferential impedance in the presence of a redox probe was in the range of 1–10 MΩ cm², too far too large for practical implementation of a glucose biosensor. The large magnitude of the interferential impedance may arise from nonspecific interactions of the protein with the sulphydryl-terminated surface, which is hydrophobic.

Another protein immobilization strategy was attempted using a maleimide linker molecule.21-23 First a cystamine SAM was formed on the Au surface, then a heterobifunctional cross-linker,
sulfosuccinimidy1-4-((N-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC), was applied. The NHS ester end of this molecule reacts with the primary amine group on the SAM, and the maleimide group is exposed, at which time the GGR A1C protein is added to the system. However, the interfacial impedance did not change significantly during this procedure, suggesting that little protein was immobilized. One possible explanation is that amine groups from the tris buffer, in which the GGR protein is stored, compete with the sulfhydryl groups to react with maleimide.25,26

Only direct protein binding to Au through the genetically engineered cysteine group provided a stable impedance value of reasonable magnitude. Figure 1 shows the Nyquist plots for the bare Au electrode, the wild-type GGR-modified Au electrode, and the GGR A1C-modified Au electrode from 1.2 to 950 Hz (10 steps per decade) at 186 mV SCE in a solution containing 100 mM KCl, 0.5 mM CaCl2, and 0.5 mM Fe(CN)6^3−/4−. These results were fit to the equivalent circuit shown in Fig. 2.  

The most critical equivalent circuit element is the charge-transfer resistance (Rct), which reflects the extent to which adsorbed protein blocks the surface. Some impedance results at Au electrodes have been reported, with the interpretation that large values of Rct indicate substantial surface coverage of protein, while values close to that for the bare Au electrode indicate little or no protein adsorption.28,29 Several authors have argued semiquantitatively that the value of Rct increases with the protein surface coverage.20-23 Such analyses have only been suggested for protein adsorption that does not involve significant charge transfer. Protein adsorption is also monitored in some cases as a reduction in the interfacial capacitance.

Here, the very similar values for Rct for the bare Au electrode and the wild-type protein suggest that protein adsorption is minimal. The dramatic increase (18×) in the charge-transfer resistance (Rct) of the GGR A1C-modified Au electrode relative to the bare Au electrode reflects the formation of a protein film on the Au electrode. The protein films dramatically slows electron transfer to Au electrode and the wild-type GGR-modified Au electrode demonstrates that the wild-type protein does not adsorb significantly onto Au electrodes from the solutions used here, as will be discussed below in further detail. The lack of protein adsorption observed here with electrochemical impedance spectroscopy is corroborated by earlier studies with a QCM.3,27 The slight differences in the impedance behavior between these two electrodes may arise from adsorption of protein fragments, which are ubiquitous for proteins stored in solution.

Table I. Value of the equivalent circuit elements in Fig. 2 for different electrode surfaces.

<table>
<thead>
<tr>
<th>Circuit Elements</th>
<th>GGR A1C</th>
<th>Wild GGR</th>
<th>Bare Au</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rct (Ω cm^2)</td>
<td>35.2 ± 0.2</td>
<td>20.9 ± 0.2</td>
<td>28.0 ± 0.2</td>
</tr>
<tr>
<td>CPE-T (μF/cm^2/10^φ^−1)</td>
<td>26.9 ± 0.23</td>
<td>24.1 ± 0.6</td>
<td>71.5 ± 2.4</td>
</tr>
<tr>
<td>CPE-b</td>
<td>0.901 ± 0.001</td>
<td>0.954 ± 0.004</td>
<td>0.886 ± 0.005</td>
</tr>
<tr>
<td>Rdl (Ω cm^2)</td>
<td>1711 ± 13</td>
<td>131 ± 1</td>
<td>97.1 ± 1.1</td>
</tr>
<tr>
<td>σ (Ω cm^2/10^φ^−1)</td>
<td>307.66 ± 17.18</td>
<td>284.57 ± 1.95</td>
<td>252.63 ± 1.16</td>
</tr>
<tr>
<td>Cdl ave (μF/cm^2)</td>
<td>56.5 ± 0.2</td>
<td>32.2 ± 0.6</td>
<td>182 ± 2</td>
</tr>
</tbody>
</table>
Faraday’s constant, respectively, electrons transferred, and constant $K$ is the stoichiometric number of electrons transferred, and $C_a$ and $C_R$ are the concentrations of the redox couple. Assuming the diffusion coefficients of $\text{Fe(CN)}_6^{3-}$ and $\text{Fe(CN)}_6^{4-}$ ($D_D$ and $D_R$) are equal, Eq. 2 and 3 yield a diffusion coefficient of $8.6 \times 10^{-9}$, and $6.7 \times 10^{-9} \text{ cm}^2 \text{s}^{-1}$ for the bare Au and the wild-type GGR-modified Au, respectively, in good agreement with other measurements. The Warburg impedance obtained for the GGR AIC-modified Au electrode is unreliable as this system is not at steady state. As seen previously, the charge transfer resistance ($R_{ct}$) increases by about 4% every 5 min.3 However, because low-frequency measurements are not involved, $R_{ct}$ can still be approximately determined for the GGR AIC-modified Au electrode.

Adsorption of a receptor protein onto a solid surface might be expected to reduce its binding affinity somewhat. In order to characterize the equilibrium binding of glucose by the GGR AIC protein immobilized onto Au, steady-state measurements are needed. As seen previously, this can be accomplished by omitting the redox probe and monitoring the impedance signal at 20 Hz in a refolding buffer containing 100 mM KCl, pH 7.1 10 mM tris buffer.7 The equilibrium impedance change arising from different glucose concentrations is shown in Fig. 3. This shows the expected saturation behavior when the glucose concentration far exceeds the equilibrium dissociation constant, which has been determined in the solution phase for the wild-type GGR protein as approximately 0.2 μM.6

The binding affinity between GGR AIC receptor protein ($P$) and glucose ($A$) can be measured by the association constant $K_a$ for the binding reaction at equilibrium8

$$K_a = \frac{P \cdot A}{[P][A]} \quad [5]$$

where $P \cdot A$ is the ligand-bound protein. However, the dissociation constant $K_d$, which is the reciprocal of $K_a$ and has units of concentration, is often a more convenient measure of binding affinity

$$K_d = \frac{1}{K_a} \quad [6]$$

Because each GGR protein molecule binds one glucose molecule, and because energetic interactions associated with glucose binding between adjacent protein molecules should be small, the glucose binding onto the GGR AIC-modified Au electrode can be treated as Langmuir adsorption at a solid surface. Therefore, with a known concentration of ligand $A$, the fraction of protein sites occupied by glucose ($\theta_A$) is determined from the Langmuir isotherm and a site balance on the immobilized protein molecule8

$$\theta_A = \frac{K_a[A]}{1 + K_a[A]} = \frac{[A]}{K_d + [A]} \quad [8]$$

The fractional change in the impedance magnitude, $y = -(\Delta Z/Z_0)$, from Fig. 3 is assumed to be directly proportional to the fraction of the immobilized protein molecules bound with glucose

$$y_{max} = \frac{[A]}{K_d + [A]} \quad [9]$$

In order to avoid overweighting the results at low glucose concentration, Eq. 10 was rearranged into the Hanes-Woolf form

$$\theta_A = \frac{y}{y_{max}} \quad [10]$$

where $y_{max}$ is the maximum fractional change in Fig. 3 when the protein surface film is saturated with glucose. Substituting Eq. 9 into Eq. 8, then fit to Eq. 11, yielding $y_{max} = 4.5(0.1)\%$ and $K_d = 6.6(1.5) \text{ μM}$, with $R^2 = 0.9956$. This equilibrium dissociation constant is about 32 times higher than the native GGR protein in solution.6 Because the introduced cysteine residue is far from the binding pocket, it should have little effect on $K_d$. Glucose binding by the GGR AIC protein may be slightly weakened by additional nonspecific interactions beyond Au–S bond formation. This value of $K_d$ for the immobilized GGR AIC is close to that (7 μm) measured by Hsieh et al. for the glucose binding protein with a cysteine mutant at the 149th amino acid position.6 However, these results are not comparable to the current study for several reasons. Hsieh et al. immobilized the GGR protein onto a standard dextran-coated SPR substrate, so no interactions with a nearby metal surface are possible.6 In addition, they introduce a hexa-histidine substitution at the C-terminus, which is much closer to the ligand binding site than the N-terminus, the point of mutation in the current studies.6 Mutations near the ligand binding site have been shown to reduce the ligand binding affinity. Our expectation is that the reduction in the binding constant obtained by Hsieh et al. may be associated with the relative proximity of the mutation site to the ligand binding site, while the reduction in the binding constant observed in the current study arises from nonspecific interactions with the underlying Au electrode. When the bare Au surface is directly exposed, image charge attractions are expected between regions of the protein surface with local charge.

![Figure 3](image-url)

Figure 3. Change in the magnitude of the impedance ($|Z|$) at 20 Hz as a function of glucose concentration at equilibrium in 10 mM pH 7.1 tris buffer containing 100 mM KCl and 0.5 mM CaCl$_2$.

$$\sigma = \frac{RT}{n^2 F^2 \sqrt{2}} \left( \frac{1}{D_D C_a(0)} + \frac{1}{D_R C_R(0)} \right) \quad [4]$$

Here, $R$, $T$, and $F$ represent the molar gas constant, temperature, and Faraday’s constant, respectively, $n$ is the stoichiometric number of electrons transferred, and $C_a$ and $C_R$ are the concentrations of the redox couple. Assuming the diffusion coefficients of $\text{Fe(CN)}_6^{3-}$ and $\text{Fe(CN)}_6^{4-}$ ($D_D$ and $D_R$) are equal, Eq. 2 and 3 yield a diffusion coefficient of $8.6 \times 10^{-9}$, and $6.7 \times 10^{-9} \text{ cm}^2 \text{s}^{-1}$ for the bare Au and the wild-type GGR-modified Au, respectively, in good agreement with other measurements. The Warburg impedance obtained for the GGR AIC-modified Au electrode is unreliable as this system is not at steady state. As seen previously, the charge transfer resistance ($R_{ct}$) increases by about 4% every 5 min.7 However, because low-frequency measurements are not involved, $R_{ct}$ can still be approximately determined for the GGR AIC-modified Au electrode.

Figure 4. Hanes-Woolf plot for the determination of the dissociation constant, $K_d$ for glucose and the immobilized GGR AIC.
and the Au surface. Such nonspecific interactions have been noted to strongly impact the relative binding orientation and surface coverage of different protein mutants.40,41 Some researchers have reported reduction in activity upon surface adsorption of proteins.42,43 For applications to glucose sensing, the increase in the dissociation constant with immobilization of GGR AIC is unimportant, because the physiologic range for glucose is approximately 1–30 mM and must be increased for technological applications anyway. In order to reach this range, the equilibrium dissociation constant can be dramatically increased by introducing residues near the ligand binding pocket that reduce this protein’s affinity for glucose.44

The use of EIS to measure the ligand equilibrium dissociation constants deserves comment. SPR spectroscopy is probably the most logical applications anyway. In order to reach this range, the equilibrium dissociation constant can be dramatically increased by introducing residues near the ligand binding pocket that reduce this protein’s affinity for glucose.45

The current results show that EIS may provide a simpler method than SPR for measuring surface-bound protein binding constant in systems where it is applicable. The only assumption is that the impedance change in Figure 3 is linearly related to the concentration of surface-bound glucose. However, it must be acknowledged that EIS will probably not be widely applicable to determining equilibrium dissociation constants of surface-immobilized proteins. Only when the protein is directly in contact with the underlying electrode, or when a sufficiently short linker molecule is employed, will the EIS signal likely vary measurably upon ligand binding.46 Both SPR methods and the current EIS studies are reagentless techniques.

The stability of the impedance signal from the Au-immobilized GGR AIC depends on the dc potential that is applied to the surface. Figure 5 illustrates the continuous drift in the impedance signal when the applied dc potential deviates from the OCP. This may reflect charging of the protein surface, which has been associated with protein denaturation.47 Thus, for the type of impedance biosensor reported previously, proper choice of the applied dc potential is important for stable operation. Alternatively, galvanostatic impedance methods might circumvent this shortcoming through application of a small ac probe current along with a zero dc current. This would effectively hold the biosensor potential close to the OCP.

Acknowledgment

This research was supported by NSF grant CTS-0329698.

Clarkson University assisted in meeting the publication costs of this article.