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A Piezoelectric Quartz Crystal Biosensor: The Use of Two Single Cysteine Mutants of the Periplasmic *Escherichia coli* Glucose/Galactose Receptor as Target Proteins for the Detection of Glucose†

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ABSTRACT: We have examined the potential utility of a glucose biosensor that employs the glucose/galactose receptor of *Escherichia coli* with a quartz crystal microbalance (QCM). Two different genetically engineered mutant proteins were utilized, each involving the incorporation of a single cysteine into the amino acid sequence of the protein. The proteins were immobilized on the surface of a piezoelectric crystal by a direct sulfur−gold linkage. Since the cysteines were located at different positions in the sequence, the receptors attach to the surface with different orientations. Considering only mass effects, the target sugars for this receptor are predicted to be too small to be detectable with a QCM. However, our sensors indicated measurable and reproducible frequency responses when immobilized receptor was exposed to sugar. This unexpectedly large frequency response occurs because the protein film is transformed from a viscous layer to a more rigid nondissipative film. The QCM can detect these changes because of the direct linkage of the proteins to the surface. Calculations of the frequency response expected for a viscoelastic film with different rheological characteristics support this hypothesis. This study is significant because it illustrates a widened applicability for the QCM methodology to protein systems that bind small molecules and undergo ligand-induced conformational changes.

Bacterial periplasmic binding proteins in Gram-negative bacteria are essential components that serve as front line receptors for a host of active transport and chemotaxis systems (*1*). These receptors function as an uptake system for sugars, anions, amino acids, and oligopeptides. Each receptor binds its target ligand(s) specifically with K_d's in the micromolar range (*2*, *3*). The receptors have monomeric structures with two distinct domains, which exhibit similar packing of secondary elements. Peptide strands, known as the hinge, link these domains. Each protein is ellipsoid in shape with the binding cleft found between the two domains. Without ligand, the domains sample a variety of conformations but are predominately open and poised to capture a ligand (*4*−*6*). Substrate binding induces a "venus fly trap" mechanism, which sequesters the ligand in the pocket, excludes the solvent molecules, induces a large conformational change in the protein, and promotes stability of the protein−ligand complex (*7*). The primary function of the conformation change is to provide a distinct form of the substrate-loaded receptor that docks to one of the two integral proteins of the inner membrane involved in chemotaxis or transport. A large network of hydrogen bonds in the binding site holds the sequestered ligand in place, stabilizes the closed form, and provides a low energy barrier for rapid ligand dissociation (*8*). This substrate binding and release mechanism and the tight specificity of binding are well documented for the two dozen or so periplasmic proteins (*9*, *10*). These characteristics along with their high solubility allow this class of receptors to be good candidates for biological recognition elements in biosensors.

Several laboratories have studied the biotechnological application of the *Escherichia coli* glucose and galactose binding protein (GGR).[1] By tagging the receptor with fluorescent probes, one detects glucose using optical methods (*11*, *12*). In addition, several groups, including our own, have investigated the use of surface plasmon resonance methods to analyze glucose binding to GGR (*13*, *14*). This receptor is an ideal biomaterial since it is structurally and functionally well characterized by a number of biophysical techniques (*4*, *15*−*17*). The receptor, which binds D-glucose with a K_d of 0.2 μM and D-galactose with a K_d of 0.4 μM, consists of 309 amino acids with a molecular mass of 33 370 Da (*18*). The protein possesses an EF-hand-like calcium-binding site that stabilizes the structure, and GGR remains functional up to 50 °C (*15*, *19*). There are no cysteine residues present in the native protein.

Our laboratory is developing a quartz crystal microbalance (QCM) technology as a biosensor for small specific ligands.

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[1] Abbreviations: GGR, *E. coli* glucose and galactose protein; GGRQ26C, *E. coli* glucose and galactose protein mutant glutamine to cysteine at position 26; GGRA1C, *E. coli* glucose and galactose protein mutant alanine to cysteine at position 1; DTT, DL-dithiothreitol; QCM, quartz crystal microbalance; Gn-HCl, guanidinium chloride; IPTG, isopropyl-β-D-thiogalactopyranoside; K_d, equilibrium dissociation constant; Da, daltons.

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Quartz crystal microbalances are suitable transducers for chemical and biochemical sensing since recent advances in overlayer preparations and transducer electronics have revolutionized the operation in liquids for studies of affinity reactions (*20*, *21*). The principle of the QCM is based on detecting the frequency shift resulting from changes on the surface of the piezoelectric crystal (*22*). Although this response is still not fully understood on the molecular level, it is recognized that the change in observed frequency is influenced by a number of interfacial properties (*23*). These include changes in mass, the effective viscosity, stiffness, conductivity, and dielectric constant of the layer, and changes in the electrode morphology due to surface roughness (*24*). The attractiveness of this technique lies in the ability to analyze reactions without labeling requirements. In addition, information on rate and equilibrium binding that describe biomolecular interactions can be investigated (*25*).

When a rigid mass is added to the surface of a piezoelectric crystal, the resulting decrease in oscillation frequency can be quantitatively interpreted using the well-known Sauerbrey equation:

$$\Delta f = -\frac{2f^2}{\sqrt{\rho_q \mu_q}} \Delta m = -C_f \Delta m \qquad (1)$$

where Δf is the frequency shift resulting from the additional mass per area, Δm, f is the intrinsic crystal frequency, ρ_q is the density of the quartz, and μ_q is the shear modulus of the quartz film. For a 5 MHz crystal, $C_f = 56.5$ Hz cm$^2/\mu$g.

When the added mass is not a rigid solid, the frequency response is dampened, resulting in a frequency shift that is less than that predicted using eq 1. When the Voight model is used to describe a viscoelastic film that is added to the piezoelectric surface, the frequency shift is related to the thickness of the film, d_f, the density of the film, ρ_f, the elastic shear (storage) modulus of the film, μ_f, and the shear viscosity (loss modulus) of the film, η_f (*26*):

$$\Delta f = \frac{f\,\mathrm{Im}(\beta)}{\pi\sqrt{\mu_q \rho_q}} + \sqrt{\frac{f^3 \rho_L \eta_L}{\pi \mu_q \rho_q}} \qquad (2)$$

where ρ_L is the density of the buffer solution, η_L is the viscosity of the buffer solution and the parameter β is defined by

$$\beta = \xi_1 \frac{2\pi n f_0 \eta_f - \mu_f i}{2\pi n f_0} \frac{1 - \alpha \exp(2\xi_1 d_f)}{1 + \alpha \exp(2\xi_1 d_f)} \qquad (3)$$

with

$$\alpha = \frac{\frac{\xi_1}{\xi_2}\frac{2\pi n f_0 \eta_f - \mu_f i}{2\pi n f_0 \eta_1} + 1}{\frac{\xi_1}{\xi_2}\frac{2\pi n f_0 \eta_f - \mu_f i}{2\pi n f_0 \eta_1} - 1} \qquad \xi_1 = \sqrt{-\frac{(2\pi n f_0)^2 \rho_f}{\mu_f + 2\pi n f_0 \eta_f i}}$$

$$\xi_2 = \sqrt{\frac{2\pi n f_0 \rho_1}{\eta_1} i}$$

The frequency shift in eq 2 represents the change in frequency expected when a viscoelastic film is added to a quartz film that is immersed in a Newtonian liquid. This change in frequency is dependent upon four characteristics of the viscoelastic film (d_f, ρ_f, μ_f, and η_f), as well as the density and viscosity of the surrounding liquid.

In previous studies involving a variety of applications, functionalization of gold surfaces was accomplished using self-assembled monolayers of thio compounds (*27*−*29*). In these studies, the sulfur was attached to the gold and the thio compounds formed a neatly ordered layer on the surface. A variety of proteins have been added to this monolayer by covalently bonding the protein to the thio compound (*30*). We have taken a new approach to forming protein monolayers on the surface. For our work, the protein is used as the functional reagent to derivatize the gold electrodes.

Two single-site mutants of GGR were genetically engineered for this study. One mutant has cysteine in the first amino acid position, the other mutant has cysteine in the 26th amino acid position. Each protein was immobilized to the electrode surface on the crystal via a sulfur−gold bond using sulfur from the newly incorporated cysteine residue. This point of attachment for both proteins is far removed from the glucose-binding site and the calcium-binding site (*17*, *19*). The frequency of the piezoelectric crystal was measured following GGR immobilization and again after exposure to sugars. Surface regeneration was examined by unfolding the protein to release sugar. Our study shows that the use of direct protein linkage to the surface expands the utility of the QCM technique, which now can be used as a biosensor for detecting small ligands. We have also demonstrated the use of the GGR−glucose system as a model to establish the versatility of periplasmic binding proteins as target proteins for regenerable biosensors.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich. 4-Fluoro-4-deoxygalactose was a generous gift from Stephen Withers, University of British Columbia (*31*, *32*). The QCM sensors employed were commercially available AT-cut piezoelectric quartz crystals (2.54 cm diameter) with 5 MHz resonant frequency. The gold electrode deposited on the crystal face that was in contact with protein and sugar solutions had 1.27 cm diameter. The crystals were provided from the manufacturer with a polished surface with surface roughness less than 50 Å. The plasmid for the GGR mutant with glutamine changed to cysteine (GGRQ26C) was obtained from Dr. Joseph Falke, University of Colorado-Boulder (*33*).

Design and Construction of the Alanine to Cysteine Mutant GGR (GGRA1C). Alanine to cysteine single amino acid substitution was introduced in the pSF5 GGR construct using Strategene QuikChange site-directed mutagenesis kit with *Pfu* turbo DNA polymerase and the oligonucleotides described below. Automated DNA sequencing confirmed the mutation.

Wild-type sequence, 5′-GCCGCTGCACACGCT [GCT] GATACTCG-3′.

Primer I, 5′-GCCGCTGCACACGCT [TGT] GATACTCG-3′.

Primer II, 5′-CGAGTATC [ACA] AGCGTGTGCAGCGGC-3′.

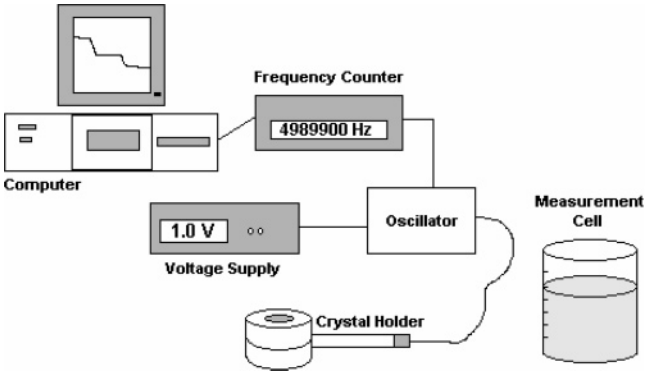

FIGURE 1: Schematic of a QCM crystal in its holder and associated electronic components for frequency acquisition.

Expression and Purification of GGRA1C and GGRQ26C. The GGRA1C and GGRQ26C plasmids were expressed in *E. coli* BL21 cells and purified as described previously (*16*, *17*). Protein was then dialyzed against two changes of 250 mL of 3 M GnHCl, 100 mM KCl, 20 mM EDTA, and 10 mM Tris, pH 7.1, and four changes of 500 mL of refolding buffer containing 100 mM KCl, 10 mM Tris, pH 7.1, and 0.5 mM $CaCl_2$. Quantitation of the proteins was determined by A_{280}.

QCM Measurement Set Up. The measurement set up consisted of a QCM (Maxtek, Inc.), which was comprised of the QCM sensors, the oscillating circuit, the frequency counter, and the personal computer shown in Figure 1. The crystal was positioned in a Teflon holder and was then fixed in position by an O-ring so that only one side of the crystal was exposed to protein or buffer solution. A 250 mL beaker containing 200 mL of 100 mM KCl, 10 mM Tris, pH = 7.1, and 0.5 mM $CaCl_2$ served as the "measurement cell". The cell was elevated on a Styrofoam platform, which provided insulation and reduced external noise interference. A small well ($\sim$500 μL) was created above the crystal surface because of the Teflon ring that was part of the crystal holder.

Attachment of GGRA1C to Au and Piezoelectric Detection of Ligand Binding. The crystal holder with a clean quartz film was immersed into the liquid environment of the "measurement cell" until a stable resonant frequency was observed and recorded. Frequency measurement was then temporarily halted. The holder was removed from the measurement cell and 400 μL of 27 μM GGRA1C protein in 100 mM KCl, 10 mM Tris, pH 7.1, and 0.5 mM $CaCl_2$ was added to the well above the crystal surface and incubated for 1.5 h. The protein immobilization method was based on the formation of a direct covalent bond between the thiolated group of the mutant cysteine and the gold electrode on the crystal. A schematic representation of protein binding to the surface is shown in Figure 2A. After incubation, the protein solution was removed from the well, and the surface was washed several times with buffer to disrupt any nonspecific binding of proteins to the surface.The holder was then returned to the "measurement cell" where frequency measurements were resumed until a stable reading was achieved. The crystal holder was removed from the "measurement cell", and a 400 μL quantity of 0.27 mM ligand in 100 mM KCl, 10 mM Tris, pH 7.1, and 0.5 mM $CaCl_2$ was added to the well above the immobilized protein on the crystal surface. Ligand concentrations were greater than 10 times the concentration of the protein solution used during

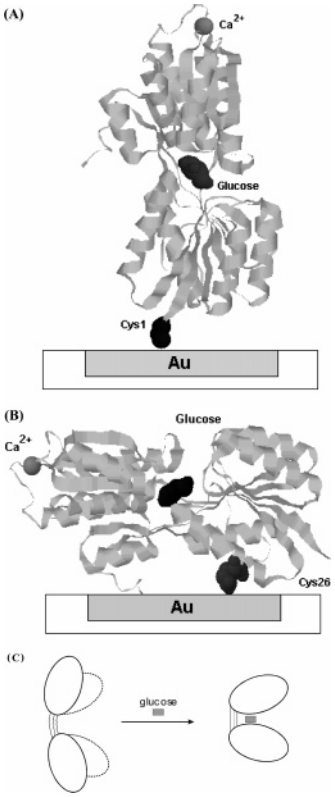

FIGURE 2: α-Carbon backbone structure of GGRA1C (A) and GGRQ26C (B) illustrating the Ca^{2+}, glucose, and attachment site to the gold surface of the piezoelectric crystal. Panel C is a cartoon depicting the change in conformation of GGR resulting from glucose binding.

receptor immobilization. Ligand was exposed to the surface for 1 h before the crystal was returned to the measurement cell and frequency was again measured to detect ligand binding. Analogous experiments were conducted for each of the five ligands: D-glucose, D-galactose, 4-fluorogalactose (*18*), fructose, and maltose. Fructose and maltose served as negative controls.

Several experiments were also performed where the GGRA1C was incubated with glucose prior to immobilization to the gold surface. The solution containing GGRA1C with bound glucose was incubated in the well above the crystal surface for 1.5 h. Following incubation, the frequency of the crystal was measured as described above.

Attachment of GGRQ26C and subsequent binding of glucose was performed under the same conditions as those for GGRA1C.

Procedure for Biosensor Regeneration. Following protein attachment, glucose binding and frequency measurements, three changes of 400 μL of 3 M guanidine−HCl were added to the crystal to partially unfold the protein and dissociate the glucose from the complex. The protein was then refolded by addition of 100 mM KCl, 10 mM, Tris pH 7.1, and 0.5 mM $CaCl_2$, and frequency shifts were recorded. Glucose was then added to the immobilized protein with free binding sites, and frequency measurements were subsequently recorded. This procedure was repeated two times.

RESULTS AND DISCUSSION

Protein Biochemistry. For the GGRA1C mutant, DNA sequencing confirmed an alanine to cysteine mutation in

Table 1: Frequency Shift Changes with Receptor Immobilization and Sugar Binding[a]

experiment		frequency shift (Hz)	
protein receptor	ligand	receptor attachment	ligand binding
GGRA1C	glucose	−38	−12
GGRA1C	glucose	−41	−13
GGRA1C	galactose	−35	−8
GGRA1C	galactose	−38	−10
GGRA1C	4-fluorogalactose	−30	−9
GGRA1C	maltose	−40	−1
GGRA1C	fructose	−39	0
GGRA1C−glucose complex		−49	
none	glucose		0
GGRQ26C	glucose	−29	−8
GGRQ26C	glucose	−33	−9

[a] Standard deviation in each measurement is ±1 Hz.

position 1 on the GGR protein. This is the sole cysteine in the protein and is found at the globular periphery of the protein structure, as shown in Figure 2A. The cysteine is located at the end of an α helix at the N-terminus of the protein. This amino acid is completely solvent-exposed, leaving the sulfur on this amino acid available to bind to the gold surface. This site was selected to attach the protein to the gold in a position that should not interfere with the glucose-binding pocket or any area involved in the ligand-induced conformational change. Since the mutation of alanine to cysteine is engineered in position 1, which is similar to the end of a "tail", this conservative change is not expected to significantly affect the structural integrity of the protein.

From crystallography measurements, it has been determined that the glucose-bound protein is ellipsoid in shape with a 35 Å minor axis and a 65 Å major axis, as shown in Figure 2A (*19*). The Au−S bond is expected to form so that the long axis of the protein should be perpendicular to the surface. Therefore, the protein should cover an area with a diameter of approximately 35 Å. Assuming that the proteins are uniformly close packed, calculations from the dimensions of the protein and the surface area of the electrode indicate that $\sim 1.11 \times 10^{13}$ protein molecules should be immobilized on the crystal surface.

GGRQ26C has the cysteine on the surface of an α helix. This cysteine is on the backside of the glucose binding pocket, and this mutant has been fully characterized for its binding capacity for sugar. (*34*) A gold−sulfur bond from this position should place the protein in a different orientation on the crystal surface compared to GGRA1C as shown in Figure 2B. If we assume that the GGRQ26C receptor occupies an area of 35 Å × 65 Å, it is predicted that $\sim 5.58 \times 10^{12}$ molecules will bind to the electrode surface.

Loading of the GGRA1C on the Surface of the Piezoelectric Crystal. The results from QCM experiments involving the immobilization of GGRA1C to the piezoelectric surface are summarized in Table 1. Observed frequency shifts ranged from 30 to 40 Hz with an average frequency shift of 37 Hz. Using this value for Δf in eq 1 yields $\Delta m = 0.66\ \mu g/cm^2$ or 1.51×10^{13} molecules of GGRA1C deposited on the surface. This value is 36% larger than the value of 1.11×10^{13} molecules predicted from geometric arguments. If the protein film is not rigid but rather flexible and dissipative, the amount of GGRA1C on the surface is predicted to be even greater than 1.51×10^{13} molecules. Several possible explanations were considered for the fact that more protein is immobilized on the surface than predicted from a geometric analysis.

It is unlikely that multilayers of protein form on the quartz surface because it is expected that any protein adsorbed above a monolayer would be removed when the quartz film was washed with buffer following exposure to receptor before resuming frequency measurements. Because the gold−sulfur bond between protein and the electrode forms with the cysteine in position 1 on the GGR, it is reasonable to expect that this "tail" configuration would enable closer packing than predicted from a rigid ellipsoid model. It is also possible that some water will be adsorbed on the exterior of the immobilized receptor molecules. A calculation of the amount of water that would need to be attached to the surface to explain the observed frequency response indicates that ∼2.5 layers of water surrounding each protein molecule would explain the observed discrepancies between predicted and measured protein mass. We speculate that some combination of these two explanations is likely responsible for our observations.

Addition of Sugars to the Immobilized GGRA1C. Following exposure to sugar solution, an additional frequency shift (relative to the frequency value of the crystal with bound GGRA1C) was observed with values summarized in Table 1. Exposure of the receptor to glucose and galactose results in a significant decrease in frequency, a response that is not unexpected because these are the natural ligands for this protein. 4-Fluoro-4-deoxygalactose shows a decrease in frequency, a response that is consistent with previous observations that this sugar also binds to GGR (*18*). When the receptor GGRA1C was incubated with glucose prior to immobilization on the crystal surface, a frequency response of 49 Hz was observed upon attachment of the GGRA1C−glucose complex to the surface, as listed in Table 1. The frequency response observed upon immobilization of the complex is the same as the net frequency change when the receptor and glucose are bound independently. Exposure of the immobilized GGRA1C to maltose and fructose results in no change in frequency, indicating that these sugars do not bind to GGR.

An experiment was conducted where a clean crystal (without bound protein) was exposed to glucose solution. No frequency change was observed, indicating that interaction of the glucose with the immobilized receptor is responsible for the frequency shift observed when glucose is introduced to the crystal with adsorbed receptor. In addition, attempts to immobilize native GGR devoid of cysteine residues also yielded no change in frequency.

Because it is known that only one glucose molecule will bind with each GGR, the frequency shift expected from glucose binding can be calculated using eq 1. These calculations yield an expected frequency shift of only 0.15 Hz when 1.11×10^{13} GGR molecules are immobilized on the surface. This value is 100 times smaller than the measured frequency shift and comparable to the noise of the QCM frequency counter. These calculations indicate that one should not be able to "see" glucose binding, but in fact, a significant and reproducible frequency response is observed.

We propose that an explanation for this discrepancy between the observed frequency shift and that predicted from the Sauerbrey equation lies in a conformation change undergone by the protein during glucose binding. When just

the receptor is immobilized on the surface, it is in an "open" position that can be described as a dumbbell with a flexible hinge between the two sections, as illustrated in Figure 2C. We speculate that this configuration leads to viscous dampening of the crystal frequency because of the flexibility between the two parts of the protein. When the protein binds with glucose, its conformation changes significantly as the two receptor domains capture the glucose molecule. The receptor with bound glucose is expected to be a more compact and structurally rigid protein layer because the hinge between the two domains must lose its flexibility as glucose is bound, as illustrated in Figure 2C. Therefore, we hypothesize that the unexpectedly large frequency shift observed with glucose binding arises from an increase in the rigidity of the receptor film. It is important to emphasize that the QCM can detect this change in conformation because the protein is directly attached to the electrode film. This hypothesis is supported using calculations based on changes in the properties of the receptor film, as well as from atomic force microscopy (AFM) measurements.

Höök and Kasemo (*26*) carried out a study of the adsorption of mussel adhesive protein to a gold electrode surface using a QCM-D technique that involves measurement of the dissipation, as well as the frequency, of the oscillating crystal at multiple harmonics. Frequency and dissipation were measured as the protein adsorbed to the gold surface, as well as following cross-linking of the adsorbed protein using $NaIO_4$. This collection of measurements provided sufficient information to enable these researchers to interpret the results in terms of a viscoelastic model for the protein films and to determine the viscoelastic characteristics of the protein film before and after cross-linking. Using assumed values for the film density ($\rho_f = 1040$ kg/m^3 for the protein film prior to cross-linking and $\rho_f = 1180$ kg/m^3 for the protein film after cross-linking), the best fit values for the film characteristics showed a significant decrease in film thickness and an increase in both the shear modulus and the shear viscosity upon cross-linking. The increases in shear modulus and shear viscosity upon cross-linking are consistent with a change from a strongly hydrated, hydrogel-like structure to a more compact protein film. The film thickness for the protein layer prior to cross-linking was in good agreement with the value determined from ellipsometry measurements. The film thickness after cross-linking was in good agreement with the value predicted from the Sauerbrey equation (eq 1), as expected for a rigid, nondissipative film.

We have used the viscoelastic properties reported by Höök and Kasemo (*26*) to examine the characteristics of the GGR films because our instrument cannot provide dissipation or frequency values at multiple harmonics. Our objective was to see whether reasonable parameter values can explain the measured frequency shifts and whether those parameter values are qualitatively consistent with our understanding of the changes undergone by GGR during glucose binding. We have considered the film properties that would yield a frequency shift of 37 Hz when a relatively soft, viscous film attaches to the quartz crystal and a frequency shift of 49 Hz (37 Hz for GGR plus 12 Hz when glucose attaches) when that film becomes more rigid and compact.

In this analysis, we have assumed a film density of 1100 kg/m^3 for the GGR alone and a density of 1300 kg/m^3 for the GGR−glucose complex. From surface plasmon resonance (SPR) (*35*) and AFM studies in our laboratory, an increase in density is expected upon glucose binding as water is released from the "hinge" region as the two domains position around the glucose molecule. From the known structure of GGR and the known point of attachment of the protein to the piezoelectric surface, we can expect the film thickness to be approximately 65 Å when glucose is bound and small angle X-ray data suggest that the film should be ~10 Å longer when there is no ligand present (*6*). It seems reasonable to expect that the shear modulus and the shear viscosity of the ligand-free GGR should be reasonably close to the values measured by Höök and Kasemo for the mussel adhesive protein before cross-linking ($\mu_f = 6.6 \times 10^4$ N/m^2, $\eta_f = 0.0018$ N). While the GGR in our experiments does not become cross-linked, we expect that the increase in rigidity of the individual receptor molecules that occurs with the addition of glucose will inhibit the ability of the receptor molecules to move independently. Therefore, we anticipate that the GGR−glucose complex will behave in a manner similar to a cross-linked film. Therefore, the modulus and viscosity of the GGR−glucose complex should be reasonably close to the values measured for the mussel adhesive protein after cross-linking ($\mu_f = 30 \times 10^4$ N/m^2; $\eta_f = 0.006$ N). Using eq 2, we can predict the frequency shift resulting from the attachment of a viscoelastic film of given properties to a piezoelectric quartz film. Our approach has been to determine the film thickness that would yield the measured frequency shift. When $\mu_f = 6.6 \times 10^4$ N/m^2, $\eta_f = 0.0018$ N, and $\rho_f = 1100$ kg/m^3, a film thickness of ~76 Å is needed to generate a frequency shift of 37 Hz upon attachment. This thickness is consistent with the predicted length of the open form of the protein. When $\mu_f = 30 \times 10^4$ N/m^2, $\eta_f = 0.006$ N, and $\rho_f = 1300$ kg/m^3, a film thickness of ~69 Å is needed to generate a frequency shift of 49 Hz upon attachment. This thickness is quite close to the 65 Å thickness that is predicted from the known structure of the receptor with bound glucose.

The above analysis shows that the frequency shifts measured with GGR immobilization and subsequent glucose binding are consistent with a model that considers the ligand-free GGR to be a relatively viscous film that leads to a dampening of the frequency response when the GGR is adsorbed. Upon glucose binding, this viscous film becomes more compact and rigid with thickness smaller than that found to describe the ligand-free GGR.

Immobilization of GGRQ26C and Binding of Glucose. The results from two QCM experiments involving the immobilization of GGRQ26C to the piezoelectric surface are included in Table 1. Observed frequency shifts were found to be smaller than those measured with the GGRA1C mutant with an average frequency shift of 31 Hz. Using this value for Δf in eq 1 yields $\Delta m = 0.55$ μg/cm^2 or 1.26×10^{13} molecules of GGRQ26C deposited on the surface.

As observed with GGRA1C, the number of receptor molecules on the surface calculated from observed frequency shifts is found to be larger than the value calculated from geometric arguments. Examination of Figure 2B shows that the receptor could plausibly occupy a smaller area with the long axis of the protein forming a nonzero angle with the surface.

From mass considerations alone, a calculation of the frequency shift expected from glucose binding to the immobilized GGRQ26C shows an expected response that is

100 times smaller than the measured value of 9 Hz. Again, we speculate that the unexpectedly large frequency response to glucose can be attributed to a conformational change in the protein that occurs with glucose binding. This hypothesis is again supported by calculations based on the Voight model of viscoelasticity.

In these calculations, we have assumed the same protein densities as were used for our calculations with the GGRA1C mutant, 1100 kg/m^3 for the ligand-free protein and 1300 kg/m^3 for the GGR−glucose complex. Our objective was to examine the film characteristics needed to explain a frequency shift of 31 Hz for a viscous, flexible film and 40 Hz for a more rigid, compact, and less flexible film. Using eq 2, our calculations indicate that attachment of a viscoelastic film with thickness 56 Å, shear viscosity of 0.0018 s N/m^2, and an elastic shear modulus of 10×10^4 N/m^2 would yield a frequency shift of 31 Hz. The smaller effective film thickness for the GGRQ26C when compared to the 76 Å thickness found to describe the characteristics of the GGRA1C is consistent with our picture of the orientation of the two immobilized mutants on the surface, as depicted in Figure 2A,B. The shear viscosity is the same value estimated for the glucose-free GGRA1C, but the shear modulus is about 50% higher than the value used to describe the GGRA1C. A comparison of the immobilized receptors, as illustrated in Figure 2A,B, indicates that the GGRQ26C should be less flexible than the GGRA1C because its attachment point is closer to the center of the receptor. The more constrained position of the GGRQ26C is consistent with a larger value for the shear modulus.

A similar analysis of the GGR−glucose complex shows that a frequency shift of 40 Hz is predicted for a viscoelastic film that is 57 Å thick with a shear viscosity of 0.006 s N/m^2 and an elastic shear modulus of 30×10^4 N/m^2. Because of the orientation of the GGRQ26C receptor on the surface, it is not surprising that our calculations indicate that the effective thickness of the protein film remains essentially constant upon glucose binding. The relatively large shear viscosity and elastic shear modulus for this film indicates that the GGR−glucose complex is relatively rigid and nondissipative, that is, not viscous, the same conclusion drawn from the results for the GGRA1C mutant.

Because our instrument is only capable of monitoring frequency at a single harmonic, it was not possible for us to collect the information needed to determine accurate values for the film characteristics for the GGR receptor with and without glucose. However, our calculations show that the measured frequency shifts are consistent with a significant change in the conformation of the receptor that occurs with glucose binding. Our calculations indicate that the ligand-free GGR forms a flexible, viscous film that leads to significant dissipation of the crystal oscillation. This film becomes considerably more rigid and nondissipative when glucose is bound. This picture is consistent with the present understanding of these receptors gained from both X-ray crystallography and surface plasmon resonance studies. As with many receptors, the bound form is more stable and less flexible since the complex must conform to a specific structure to trigger subsequent events in the transport or chemotaxis pathways. It is this conformational change that enables the QCM to serve as a glucose sensor.

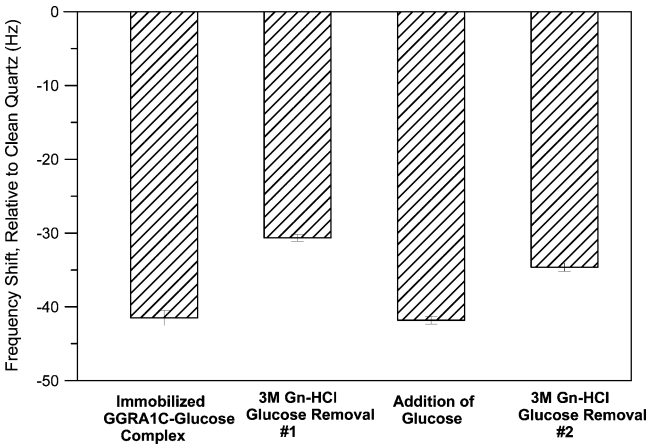

FIGURE 3: Frequency response illustrating regeneration of the GGRA1C binding sites. Regeneration of the binding sites was accomplished by incubating the immobilized protein with 3 M Gn-HCl, which partially unfolds the protein releasing glucose. The protein was then refolded using buffer containing 100 mM KCl, 10 mM Tris, pH 7.1, and 0.5 mM $CaCl_2$. The response to sequential steps is shown. Error bars represent the standard deviation in each measurement.

Glucose Binding Site Regeneration. The regeneration of binding surfaces is important for reusing biosensors and is difficult to achieve in many protein systems. The ability of the GGR to bind and release ligand allows our system to be regenerated with ease. A common procedure to rid receptors of ligands is to partially unfold the protein with subsequent washing with refolding buffer. The refolding buffer allows the protein to reassemble into its native form. This is usually performed in dialysis tubing or concentrating devices and is quite successful in removing substrates that bind in the micromolar range (*16*).

Because the receptor is bound to the crystal surface in our study, immobilized GGRA1C could be regenerated without standard devices such as dialysis tubing. We have examined the frequency response of the receptor upon exposure of the immobilized GGRA1C−glucose complex to 3 M Gn-HCl. Our objective with this treatment was to partially unfold the surface bound protein, releasing glucose without breaking the sulfur−gold bond. The surface was then rinsed with an excess amount of buffer (100 mM KCl, 10 mM Tris, pH 7.1, 0.5 mM $CaCl_2$) to foster the refolding of the GGR. The extent of refolding was examined by reintroducing glucose to the regenerated immobilized receptor. Results from several regeneration cycles are shown in Figure 3. Upon exposure of the GGRA1C−glucose complex to Gn-HCl, the frequency increased 11 Hz, indicating a release of sugar. Exposure to buffer showed that the protein was refolded and functional since the later addition of glucose resulted in a decrease in the quartz crystal frequency, returning the system to its prior frequency with glucose. Apparently, the immobilized GGR layer was not damaged by this method because this regeneration cycle was reproducible for two cycles of binding and regeneration without detectable loss of sensitivity.

CONCLUSION

The potential utility of a quartz crystal microbalance based glucose biosensor has been demonstrated. This biosensor uses a genetically engineered glucose receptor that is directly

bound to the piezoelectric crystal. Significant and reproducible frequency shifts result from protein attachment to the gold electrode, as well as subsequent glucose binding to the receptor. The magnitude of these frequency shifts was interpreted using a Voight model of a viscoelastic film. These calculations indicate that the receptor undergoes a significant change in conformation with glucose binding. Film characteristics show that the glucose-free protein layer is quite viscous with dampening of the crystal oscillation. The film becomes considerably more rigid when glucose is bound to the receptor. Glucose is a small ligand that is not expected to produce a measurable frequency response with a QCM if one only considers mass effects. Our results illustrate that a QCM-based glucose sensor is possible because the QCM is not just a mass sensor but detects ligand-induced structural changes in rigidity. This expands the utility of this method considerably since there are many proteins that bind low molecular weight ligands and undergo subsequent conformational changes. Efforts are continuing in our laboratory to further the methodology by incorporating a flow cell system through which the proteins can be absorbed and washed, analytes added, and proteins regenerated. In addition we are applying the method to other receptor proteins in the steroid family. In summary, the results we presented here show great promise for the use of QCM with immobilized periplasmic binding proteins as biosensors for a large repertoire of small ligands.

Our methodology reveals a new concept for the development of small molecule detection by receptors and the development of a high-throughput screening methods for small ligands. We believe the direct protein linkage to the surface will expand the range of QCM applications to include the many proteins that change conformation upon ligand binding. Included in this family of receptors are the important metal binding proteins and steroid receptors.

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