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Glucose Sensor for Low-Cost Lifetime-Based Sensing Using a Genetically Engineered Protein¹

Leah Tolosa^{*}, Ignacy Gryczynski^{*}, Lisa R. Eichhorn[†], Jonathan D. Dattelbaum^{*}, Felix N. Castellano^{*}, Govind Rao[†], Joseph R. Lakowicz^{*,2}

^{*}School of Medicine, University of Maryland, 725 West Lombard Street, Baltimore, Maryland 21201

[†]Department of Molecular Biology & Biophysics and Department of Chemical and Biochemical Engineering, University of Maryland, 725 West Lombard Street, Baltimore, Maryland 21201

Abstract

We describe a glucose sensor based on a mutant glucose/galactose binding protein (GGBP) and phase-modulation fluorometry. The GGBP from *Escherichia coli* was mutated to contain a single cysteine residue at position 26. When labeled with a sulfhydryl-reactive probe 2-(4'-iodoacetamidoanilino)naphthalene-6-sulfonic acid, the labeled protein displayed a twofold decrease in intensity in response to glucose, with a dissociation constant near 1 μ M glucose. The ANS-labeled protein displayed only a modest change in lifetime, precluding lifetime-based sensing of glucose. A modulation sensor was created by combining ANS26-GGBP with a long-lifetime ruthenium (Ru) metal-ligand complex on the surface of the cuvette. Binding of glucose changed the relative intensity of ANS26-GGBP and the Ru complex, resulting in a dramatic change in modulation at a low frequency of 2.1 MHz. Modulation measurements at 2.1 MHz were shown to accurately determine the glucose concentration. These results suggest an approach to glucose sensing with simple devices.

Diabetes results in long-term health consequences, including cardiovascular disease and blindness. These adverse health effects have resulted in a worldwide effort to develop noninvasive methods to monitor blood glucose. A wide variety of methods have been proposed, including near-infrared spectroscopy (1–4), optical rotation (5, 6), and amperometric (7, 8), colorimetric (10, 11), and fluorescence detection (12–17). Despite intensive efforts, no method is presently available for noninvasive measurement of blood glucose.

In this preliminary report we described a new fluorescent method to measure glucose. The method is capable of measuring micromolar glucose concentrations without reagent consumption. This high sensitivity to glucose suggests the possibility of measuring the low glucose concentrations known to be present in extracted interstitial fluid (18). Such samples are known to be painlessly available using methods which perturb the outermost layer of skin, the stratum corneum. These methods include laser ablation (19) and weak suction (20).

²To whom correspondence should be addressed.

¹Dedicated to Dr. Henryk Szmazinski on the occasion of his 50th birthday.

Our glucose sensor is based on the glucose/galactose binding protein (GGBP)³ from *Escherichia coli* (21). This protein binds glucose with a dissociation constant near 0.8 μM (21, 22). This protein and similar transport proteins from other bacteria are highly specific for binding glucose and/or galactose. The binding affinity for other sugars is typically 100- to 1000-fold weaker for sugars other than glucose or galactose (22–25). The structural studies of GGBP reveal two domains, the relative positions of which change upon binding of glucose (26) (Scheme 1). GGBP contains numerous surface lysine residues and is thus not easily labeled at a specific location. We created a single cysteine mutant of GGBP by replacement of the amino acid residue at position 26 with a cysteine. This residue was labeled with the environmentally sensitive probe 2-(4'-iodoacetamidoanilino)naphthalene-6-sulfonic acid, sodium salt (I-ANS). The emission intensity of the labeled protein ANS26-GGBP was found to be sensitive to glucose. The intensity changes of the ANS probe decreased about twofold on glucose binding, with an apparent dissociation constant near 1 μM glucose. The glucose-sensitive intensity of ANS26-GGBP allowed us to design a sensor using ANS26-GGBP and a long-lived metal–ligand complex. For the combined emission of ANS26-GGBP and the metal–ligand complex the modulation of the emission, at low frequencies, depends on the fractional fluorescence intensity of ANS26-GGBP. The fractional intensity decreases on binding glucose, resulting in a decrease in the modulation that can be used to measure the glucose concentration.

MATERIALS AND METHODS

Fluorescence Measurements

Frequency–domain intensity decays were measured with instrumentation described previously (27) and modified with a data acquisition card from ISS, Inc. (Urbana, IL) (28). Excitation was at 325 nm from a HeCd laser modulated with a Pockels cell. For the long-lifetime fluorophore we used $[\text{Ru}(\text{bpy})_3]^{2+}$ in polyvinyl alcohol. A variety of long-lifetime metal–ligand complexes are now available (29–32). For construction of the sensor $[\text{Ru}(\text{bpy})_3]^{2+}$ was dissolved in heated polyvinyl alcohol, which was then painted on the outside of the cuvette, which contained the glucose-sensitive protein (ANS26-GGBP).

Emission spectra were recorded on an Aminco SLM AB2 spectrofluorometer using an excitation wavelength of 325 nm. Polarizers were used to eliminate the effect of Brownian rotation. The concentration of ANS26-GGBP was 0.25 μM . The fluorescence spectra are relative to an identical reference sample that was sugar-free.

Construction of the Q26C Mutant of GGBP

The *mgIb* gene that encodes for wild-type GGBP and its natural promoter were isolated from the *E. coli* K-12 genome and amplified by PCR. The gene-promoter fragment was inserted into the *Pst/SstI* restriction sites of the pTz18U phagemid (Bio-Rad, Hercules, CA). The resulting plasmid, pJLO1, was used as template for the construction of the Q26C mutant. Site-directed mutagenesis was accomplished using the Quick-Change mutagenesis

³Abbreviations used: ANS26-GGBP, cysteine 26 mutant GGBP labeled with I-ANS; GGBP, glucose/galactose binding proteins; I-ANS, 2-(4'-iodoacetamidoanilino)naphthalene-6-sulfonic acid, sodium salt; $[\text{Ru}(\text{bpy})_3]^{2+}$, ruthenium tris(2,2'-bipyridine)dichloride; TCEP, tris(2-carboxyethyl)phosphine; LED, light-emitting diode; GFP, green fluorescent protein.

kit from Stratagene. The DNA sequencing data verified the presence of the desired point mutation (Biopolymer Core Facility, University of Maryland, Baltimore, MD).

Isolation of Q26C GGBP

The monocysteine mutant of GGBP was overproduced in *E. coli* NM303 (F^+ *mgI* 503 *lacZ* *lacY*⁺ *recA1*), a mutant strain that does not produce GGBP. The cultures consisted of 0.5% inoculum, 25 μ g/ml ampicillin in 200 mL Luria-Bertani (LB) medium (10 g/liter bacto-tryptone, 5 g/liter bacto-yeast extract, 10 g/liter NaCl, pH 7.2) and 1 mM fucose incubated in a 1-liter shake flask at 37°C and 260 rpm. Cells were harvested at 16 h, and GGBP was extracted by osmotic shock as previously described (33). The crude extract was resuspended in concentrated Tris-HCl and EDTA buffers so that the final concentration was 5 and 1 mM, pH 8.0, respectively. The GGBP cysteine mutants also received a final concentration of 1 mM tris(2-carboxyethyl)phosphine (TCEP). The GGBP was purified based on a previous method (34) using a DEAE anion-exchange column (Bio-Rad). GGBP was eluted with a 5 mM Tris-HCl, pH 8.0, gradient from 0 to 0.5 M NaCl. Fractions were analyzed for the presence of GGBP by SDS-PAGE, and fractions containing GGBP were pooled and concentrated (Amicon, 10 K MWCO). Fraction purity was analyzed by total protein (Sigma Chemical Co., St. Louis, MO) and SDS-PAGE. Image analysis was performed using an Alpha Imager 2000 analysis system (Alpha Innotech Corp.). Samples from the crude extract and DEAE were analyzed for purity on SDS-PAGE using green fluorescent protein (GFP) as calibration standards.

Labeling of Q26C GGBP with I-ANS

A solution containing 2.5 mg/ml Q26C GGBP in 20 mM phosphate, 1 mM TCEP, pH 7.0, was reacted with 50 μ L of a 20 mM solution of I-ANS in tetrahydrofuran (purchased from Molecular Probes, Inc.). The resulting labeled protein was separated from the free dye by passing the solution through a Sephadex G-25 column. The protein-ANS conjugate was purified further on Sephadex G-100.

RESULTS

Emission Spectra of ANS-GGBP

Emission spectra of ANS26-GGBP are shown in Fig. 1. Addition of micromolar concentrations of glucose resulted in an approximately twofold decrease in the intensity of the ANS label. ANS is known to be sensitive to its local environment, with lower intensities in more polar environments (35). The decrease in intensity suggests that ANS is displaced into the aqueous phase upon binding of glucose to ANS26-GGBP. This is consistent with the glucose-bound structure of GGBP (Scheme 1), where the residue on position 26 is pointing toward the aqueous phase. Similar results were obtained with other single cysteine mutants of GGBP (21). In the absence of glucose, the GGBP is known to become more flexible (26). It is likely that the flexibility of the protein in the absence of glucose allows ANS to position itself in a more hydrophobic surface of the protein, resulting in increased fluorescence intensity.

Frequency-domain intensity decays were also measured (not shown). The intensity decay of the ANS label was found to be mostly unchanged by glucose. The absence of a significant change in lifetime was initially disappointing since one of our goals is a glucose sensor based on lifetime. Nonetheless, the spectra shown in Fig. 1 demonstrate the possibility of measuring micromolar glucose concentrations with this mutant of GGBP if one can use the change in intensity.

Simulations of a Modulation-Based Glucose Sensor

In previous studies of light quenching we examined intensity decays of fluorophore mixtures with widely spaced lifetimes (36). These data indicated that the modulation was highly sensitive to the fractional intensity of the short- and long-lifetime species in the mixture. Hence, we designed a sensing strategy based on the changing modulation due to intensity changes of the shorter lifetime concept. Such changes in intensity occur for many fluorophores in response to analytes (37) and were observed for labeled GGBP upon glucose binding (Fig. 1).

Simulated frequency-domain data for a mixture of fluorophores are shown in Fig. 2. The lifetimes were assumed to be $\tau_1 = 5$ ns and $\tau_2 = 1000$ ns = 1 μ s. The lifetime of 5 ns is comparable to the mean lifetime of ANS-GGBP. Metal-ligand Re complexes with lifetimes of more than 1 μ s are now available (29–32), so that 1- μ s fluorophores are available. For these simulations we assumed the fractional intensity of the 5-ns component changed from 0.1 to 0.76. There is a region near 2 MHz where the modulation is almost independent of modulation frequency. Importantly, the modulation is sensitive to the fractional intensity of the short-lived component. For the assumed lifetimes the modulation at 2 MHz is essentially equal to the amplitude of the short-lived component. This is shown in Fig. 3, which indicates that the modulation at 2 MHz is nearly equivalent to the fractional amplitude of the short-lifetime component. This result can be easily understood by noting that the modulation of the 5-ns component is near 1.0 at 2 MHz, and the modulation of the 1- μ s component is near zero at 2 MHz. The theory describing such modulation sensors is described in a more detailed paper on this topic (38).

What accuracy in glucose concentration can be expected for such a modulation sensor based on a mixture with lifetime of $\tau_1 = 5$ ns and $\tau_2 = 1$ μ s? To answer this question we calculated the changes in modulation which could be expected for the twofold intensity changes displayed by GGBP (Fig. 4). For this glucose-sensitive protein the twofold decrease in intensity of GGBP could decrease the modulation of 2 MHz from 0.81 to 0.66 (Fig. 4). The modulation can be routinely measured to an accuracy of 0.005, which would result in glucose concentrations around ± 0.2 μ M. We note that a larger change in intensity of the glucose-sensitive emission would result in larger changes in modulation and higher accuracy in the glucose concentration. Also, with dedicated instruments the modulation may be measured to higher accuracy.

Modulation-Based Glucose Measurements

We used the concept described above to measure glucose. The labeled protein ANS26-GGBP was placed adjacent to the ruthenium complex to result in a fractional GGBP

intensity near 0.87 in the absence of glucose. The Ru complex was in a thin polyvinyl alcohol film outside the cuvette containing ANS26-GGBP. Frequency responses are shown in Fig. 5. These responses are comparable to the simulations shown in Fig. 2. Importantly, the modulation at 2.1 MHz decreases in the presence of glucose, as expected for decreased emission for ANS26-GGBP. These changes in modulation were used to prepare a calibration curve for glucose (Fig. 6). These data demonstrate that the ANS26-GGBP can be used to quantify micromolar concentrations of glucose. Modulation measurements accurate to $m = \pm 0.007$ would result in glucose concentrations accurate to $c = \pm 0.2 \mu\text{M}$. We expect future labeled GGBP mutants will display large changes in fluorescence and to yield more accurate glucose measurements.

DISCUSSION

The measurements described above represent our initial attempt to perform glucose sensing with an engineered protein. In the present results we physically separated the components with the short and long lifetimes. This was done to avoid interactions of the long-lived ruthenium complex with GGBP. Such a physical separation can be readily accomplished in a real-world sensor. For instance, the sensor may consist of two polymeric layers, one containing labeled GGBP and the other containing the long-lifetime complex. Alternatively, one may choose other long-lived fluorophores which do not interact with the protein, such as the highly charged ruthenium complex proposed recently as a water-soluble oxygen sensor (39).

It is interesting to consider the opto-electronics required for modulation-based sensing. It is now known that blue light emitting diodes (LEDs) can be amplitude modulated from 0.1 to 100 MHz (40). Also, LEDs with ultraviolet output near 380 nm are available and can be modulated to 100 MHz (41). The optical output of electroluminescent devices can also be modulated at MHz frequencies (42). Hence, simple inexpensive light sources could be used for a modulation glucose sensor.

A device for modulation-based sensing can be simpler than the usual phase-modulation instruments. For phase-angle measurements the detector must be modulated with a fixed-phase relationship to the modulated excitation. Modulation measurements can be performed without the phase-locked relationship, simplifying the electronics. These considerations suggest that a portable battery-powered device can be designed to monitor glucose. The sensitivity to low glucose concentrations suggests its use to monitor glucose in interstitial fluid. Because of the high affinity of GGBP for glucose, this device could be used with diluted blood, as the glucose concentration in whole blood is in the millimole range. Recent experiments show the feasibility of constructing low-cost instrumentation for phase-modulation measurements up to 100 MHz (43).

Finally, it is valuable to note that one can imagine a variety of GGBP mutants, with different fluorescent labels. Depending on the fluorophores, one can imagine changes in emission intensity, lifetime, anisotropy, or energy transfer. Additionally, one can imagine GGBP mutants with a range of glucose binding constants. Glucose sensors could be configured using more than one protein, providing accurate measurements over a wide range of glucose

concentrations. In our opinion, engineered glucose-sensitive proteins, coupled with new methods to painlessly extract interstitial fluid, provide a promising near-term method for real-time monitoring of glucose. The approach presented here may be readily extended to other analyte binding proteins, thus paving the way for a new generation of biosensors.

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FIG. 1. Emission spectra of ANS-Q26 GGBP in the presence of 0 to 8 μM glucose. [GGBP] = 0.25 μM , excitation at 325 nm. (Inset) The change in intensity versus glucose concentration.

FIG. 2.

Simulated frequency-domain intensity decays for a mixture of fluorophores, $\tau_1 = 5$ ns, $\tau_2 = 1000$ ns, $f_1 = 0.76$ to 0.1.

FIG. 3.
Simulated dependence of the modulation at 2 MHz on the fractional intensity (f_1) of the 5-ns component.

FIG. 4.
Simulated modulation for a glucose sensor with $\tau_1 = 5 \text{ ns}$ and $\tau_2 = 1000 \text{ ns}$.

FIG. 5.
Frequency responses of the glucose sensor at 0, 1, 4, and 8 μM glucose.

FIG. 6.
Effect of glucose on modulation of the glucose sensor at 2.1 MHz.

SCHEME 1.

Three-dimensional structure of the glucose/galactose binding protein showing cysteine 26 labeled with ANS and the glucose binding site. The structure is the glucose-bound form.