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A Novel Fluorescence Competitive Assay for Glucose Determinations by Using a Thermostable Glucokinase from the Thermophilic Microorganism *Bacillus stearothermophilus*

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Abstract

We describe the use of a thermostable glucokinase in a novel competitive fluorescence assay for glucose. Glucokinase from *Bacillus stearothermophilus* (BSGK) was found to retain enzymatic activity in solution for over 20 days. The single cysteine residue in BSGK, which is near the active site, was labeled with a fluorescent probe, 2-(4-iodoacetamidoanilino)naphthalene-6-sulfonic acid. The ANS-labeled BSGK displayed a modest 25% decrease in the emission intensity upon binding glucose but no change in lifetime. To obtain a larger spectral change we developed a competitive assay for glucose using the intrinsic tryptophan fluorescence from BSGK and a resonance energy transfer (RET) acceptor-labeled sugar. The sugar-labeled acceptor quenched the BSGK tryptophan emission, and the quenching was reversed upon addition of glucose. The use of RET as the sensing mechanism can be easily extended to longer wavelengths for a more practical glucose sensor.

Close control of blood glucose is essential to avoid the long-term adverse consequences of elevated blood glucose, including neuropathies, blindness, and other sequella (1, 2). Noninvasive measurements of blood glucose have been a long-standing research goal. Such a capability would immediately allow the development of a variety of devices for diabetic health care, including continuous painless glucose monitoring, control of an insulin pump, and warning systems for hyper- and hypoglycemic conditions. Hypoglycemia is a frequent occurrence in diabetics and can result in coma or death. The acute and chronic problems of diabetics and hypoglycemia can be ameliorated by continuous monitoring of blood glucose. At present the only reliable method to measure blood glucose is by a finger stick and subsequent glucose measurement, typically by glucose oxidase (3). This procedure is painful and even the most compliant individuals, with good understanding and motivation for glucose control, are not willing to stick themselves more than several times per day.

Because of the medical needs, there continues to be intensive efforts to develop sensors for glucose (5–9). The absence of a suitable noninvasive glucose measurement has resulted in

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decades of research, little of which has resulted in simpler and/or improved glucose monitoring. Included in this effort is the development of fluorescence probes specific for glucose, typically based on boronic acid chemistry (10–15). An alternative approach to glucose sensing using fluorescence is based on proteins which bind glucose.

Optical detection of glucose appears to have had its origin in the promising studies of Schultz and co-workers (16–19), who developed a competitive glucose assay which does not require substrates and does not consume glucose. This assay used fluorescence resonance energy transfer (RET)² between a fluorescence donor and an acceptor, each covalently linked to concanavalin A (ConA) or dextran. In the absence of glucose the binding between ConA and dextran resulted in a high RET efficiency. The addition of glucose resulted in its competitive binding to ConA, displacement of ConA from the labeled dextran, and a decrease in the RET efficiency. These early results generated considerable enthusiasm for fluorescence sensing of glucose (20–22).

The glucose–ConA system was also studied in this laboratory (23–25). We found that the system was only partially reversible upon addition of glucose, became less reversible with time, and showed aggregation. For this reason we explored the use of other glucose-binding proteins as sensors (26–28), an approach also used in other laboratories (29, 30). In fact, there is now considerable interest in using proteins as sensors for a wide variety of substances (31–35). If a reliable fluorescence assay for glucose could be developed, then the robustness of lifetime-based sensing (36–38) could allow development of a minimally invasive implantable glucose sensor or a sensor which uses extracted inter-stitial fluid. The lifetime sensor could be measured through the skin (39) using a red laser diode or light-emitting diode (LED) device as the light source. These devices are easily powered with batteries and can be engineered into a portable device. An implantable sensor can be expected to report on blood glucose because tissue glucose closely tracks blood glucose with a 15-min time lag (40, 41), and time delays as short as 2–4 minutes have been suggested (42).

In the present report we describe the use of a thermostable glucokinase for use as a reversible glucose sensor. We believe hexokinases have good potential for use as glucose sensors. These proteins catalyze the following results:

In the absence of ATP the protein will not carry out the phosphorylation and thus will not consume glucose.

Structure and Mechanism of Glucose Binding of Hexokinase

The structure of the hexokinase A from yeast has been determined by X-ray diffraction both in the absence and in the presence of glucose (43–46). The polypeptide chain of 485 amino

²Abbreviations used: BS, *Bacillus stearothermophilus*; ConA, concanavalin A; FD, frequency domain; GK, glucokinase; IA-ANS, 2-(4-iodoacetamidoanilino)naphthalene-6-sulfonic acid, sodium salt; ONPG, *o*-nitrophenyl- β -D-glucopyranoside; RET, resonance energy transfer; YHK, yeast hexokinase; LED, light-emitting diode.

acid residues in the yeast protein is folded into two distinct domains, a smaller N-terminal domain and a larger C-terminal domain. From the high-resolution crystal structures of the enzyme is evident that in the absence of ligand, the two domains are separated by a deep cleft. This cleft represents the enzyme active site. It is in this region that the enzyme binds the substrate. In particular, the binding of glucose causes the small lobe of the molecule to rotate by 12° relative to the large lobe, moving the polypeptide backbone as much as 8°, closing the gap between the two domains. The domain rotation has two effects: the glucose molecule is buried into the interior of the protein and the side chains in the active site are rearranged.

Fluorescence spectral data in the literature suggest that hexokinase can be used as an optical glucose sensor. For instance, glucose binding to the native dimer and monomer hexokinase from *Saccharomyces cerevisiae* was monitored by following the concomitant quenching of the protein fluorescence (47–49). This enzyme possesses four tryptophan residues that can be classified as two surface residues, one glucose-quenchable residue in the cleft and one buried. The maximal quenching induced by glucose was about 25% and the concentration of glucose at half-maximal quenching was 0.4 mM for the monomeric form and 3.4 mM for the dimeric one (50, 51).

For use as a sensor a protein should have long-term stability. Unfortunately, yeast hexokinase (52) and human hexokinase (53) are unstable and quickly lose activity at room temperature. Thermophilic microorganisms produce enzymes with unique characteristics such as high temperature, chemical, and pH stability. These enzymes are already in use as biocatalysts in industrial processes (54, 55).

A glucokinase from the thermophilic organism *Bacillus stearothermophilus* has been characterized (56, 57) and is known to display long-term stability (52). Hence we evaluated the use of this glucokinase (BSGK) in the absence of ATP as a glucose-nonconsuming glucose sensor. This protein has already been used as an active enzyme in glucose assays (58, 59).

MATERIALS AND METHODS

Materials

BSGK, yeast hexokinase (YHK), *o*-nitrophenyl- β -glucopyranoside, IA-ANS, and D-glucose were obtained from Sigma. BSGK was dissolved in 10 mM Tris-HCl buffer, pH 9.0. This enzyme solution represents the starting material for the fluorescence measurements. For all fluorescence measurements the final concentration of BSGK was 3 μ M. Steady-state measurements were performed in quartz cuvettes in an ISS spectrofluorometer using magic angle polarizer conditions.

Frequency-domain (FD) measurements were performed using instrumentation described previously (60). For 370-nm excitation the light source was a frequency-doubled pyridine-2 dye laser and the emission observed through a 465-nm interference filter. The FD measurements were performed using magic angle polarizer conditions. The FD intensity decay data were analyzed by nonlinear least squares in terms of the multiexponential model

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i), \quad [1]$$

where α_i are the preexponential factors associated with the decay time τ_i , with $\sum_i \alpha_i = 1.0$. The mean lifetime is given by

$$\bar{\tau} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} = \sum_i f_i \tau_i, \quad [2]$$

where f_i are the fractional steady-state intensities of each lifetime component

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j}. \quad [3]$$

The intensity-weighted lifetime is given by

$$\langle \tau \rangle = \sum_i \alpha_i \tau_i; \quad [4]$$

the values of $\langle \tau \rangle$ are thought to be proportional to the quantum yield of the sample.

Polarization Sensing

Polarization sensing provides a method by which a change in intensity is observed as a change in polarization. This can be accomplished using a polarized reference film (61, 62) or as in the current paper using a protein sample in the absence of analyte (63). The principle of the measurement is shown in Scheme 1. Two solutions are placed side by side. The reference (*R*) solution observed through a vertical polarizer contains the protein without any glucose. The sample (*S*) observed through a horizontal polarizer contains the protein with various concentrations of glucose. The polarization of the combined emission from the sample and reference is given by

$$P = \frac{I_{\parallel}^T - I_{\perp}^T}{I_{\parallel}^T + I_{\perp}^T}, \quad [5]$$

where the superscript *T* indicates the sum of the intensities from the sample and reference. The subscripts indicate the parallel ($\parallel$) and perpendicular ($\perp$) components of the emission as measured through the analyzer polarizer. Because of the polarizers in front of the reference and sample sides of the sensor the polarized intensities are given by

$$I_{\parallel}^T = I_{\parallel}^R \quad [6]$$

$$I_{\perp}^T = I_{\perp}^S, \quad [7]$$

where $I_{\parallel}^R$ and $I_{\perp}^S$ represent the intensities from the reference and sample, respectively. In the present case, and in many situations, the emission from the sample and reference is

unpolarized, so that the polarized intensities are proportional to the total intensity from each side of the sensor. The intensities from the sample and reference depend on a number of instrumental factors. For simplicity we chose not to explicitly indicate these factors.

Substituting of Eqs. [6] and [7] into Eq. [8] yields

$$P = \frac{I_{\parallel}^R - I_{\perp}^S}{I_{\parallel}^R + I_{\perp}^S}. \quad [8]$$

The measured polarization depends on the intensity of the sample relative to the reference. If the sample intensity is very low then the polarization approaches 1.0. If the emission from the sample dominates then the polarization approaches -1.0. Hence a wide range of polarization values is available, resulting in a wide dynamic range for the sensor. It is important to notice that polarization-based sensing can be accomplished without a change in the polarization of the sample. This result is obtained because the polarizers on the emission side of the sensor provide polarized light from sample and reference.

In order to obtain the maximum change in polarization one must select the initial relative intensities of the sample and reference. Assume the observed initial polarization P_0 is given by

$$P_0 = \frac{I_0^S - I_0^R}{I_0^S + I_0^R}. \quad [9]$$

And that the initial ratio of the sample to the reference intensity is

$$k = \frac{I_0^S}{I_0^R}. \quad [10]$$

The initial polarization of the sample will be P_0 ,

$$P_0 = \frac{k - 1}{k + 1}. \quad [11]$$

If in response to glucose the sample ($I_{\perp}^S$) intensity will change n -fold, $I_{\perp}^S = nI_0^S$, it is possible to calculate the observed total change in polarization (ΔP) as a function of values n and k .

$$\Delta P = \frac{nk - 1}{nk + 1} - \frac{k - 1}{k + 1}. \quad [12]$$

Figure 1 shows the dependence of observed polarization change ΔP as a function of initial sample to the reference intensity ratio, k , for given values of n . One may calculate the optimal initial sample to the reference intensity ratio, k , corresponding to an expected n -fold change in the sample intensity.

$$k = \sqrt{\frac{n-1}{n^2-n}}. \quad [13]$$

It is interesting to consider values of k needed to obtain the maximum change for P for different values of n . Figure 1 (bottom) shows the simulations of the optimal (corresponding to the maximal polarization change) value of k as a function of n . From this theoretical prediction, it is possible to optimize the initial experimental conditions in order to get the biggest change of polarization upon glucose addition.

As an example, assume the sample intensity increases $n = 2$ -fold due to the analyte. From examination of Fig. 1, one may expect maximum polarization change of about -0.35 for a value k near 0.7 – 0.8 . In practice, it will be optimal to set the initial fluorescence intensity of the sample close to 70 – 80% of that of reference signal.

RESULTS

For use as a sensor the protein must display good long-term stability. In order to check the stability properties of BSGK, we incubated a solution of the enzyme (enzyme concentration 1.0 mg/ml) at room temperature. Enzyme aliquots were withdrawn and the enzyme activity as well as the fluorescence intensity were monitored. Figure 2 shows the enzyme activity and intrinsic fluorescence of BSGK and yeast hexokinase over a period of incubation time at room temperature. Yeast hexokinase loses activity over several days and the fluorescence intensity simultaneously decreases. In contrast BSGK loses no activity over 2 weeks at room temperature (52) and the fluorescence intensity remains constant. Hence BSGK is a good candidate for a glucose sensing probe.

BSGK has a single cysteine residue located near the active site (66). We labeled the residue with a sulfhydryl-reactive fluorophore IA-ANS. The emission of the labeled protein was near 460 nm (data not shown). The intensity of the ANS-labeled protein decreased upon addition of glucose (data not shown). The decreased intensity is consistent with displacement of the water-sensitive ANS into the aqueous phase upon binding glucose. The change in intensity occurs near 3 mM, which is comparable to the concentration of glucose in blood. The important conclusion from these observations is that BSGK binds glucose in the absence of ATP and can thus serve as a nonconsuming glucose sensor.

For highly accurate glucose measurements we were not satisfied with the magnitude of the intensity change. We examined the fluorescence lifetimes to determine if a change occurred upon glucose binding. Unfortunately, ANS-labeled BSGK displayed no change in lifetime upon glucose binding. Hence we considered alternative methods to use BSGK as a glucose sensor.

RET reliably occurs whenever fluorescent donors and acceptors are in close proximity (65). We developed a method to use RET to develop a competitive glucose assay. To demonstrate the feasibility of a competitive glucose assay we used the unmodified protein and its intrinsic tryptophan emission as the donor. As the acceptor we used glucose containing the

absorbing nitrophenyl group, ONPG in Fig. 3. Figure 4 shows the intrinsic tryptophan emission of BSGK. Addition of ONPG (3 μ M) resulted in an approximate 80% decrease in the tryptophan intensity. Addition of glucose resulted in recovery of the fluorescence intensity. At about 6 mM glucose concentration fluorescence intensity returns to its initial value before addition of ONPG. Further addition of glucose does not change the fluorescence signal. The fact that the intensity was sensitive to glucose demonstrates that the intensity changes are due to a binding event and not due to trivial inner filter effects from ONPG.

In recent publications we addressed the problem of obtaining reliable intensity measurements for sensing which could be used in the absence of useful changes in lifetimes. Polarization sensing is accomplished by constructing a sensor such that a stable intensity reference is observed through one polarizer and the sample is observed through a second orthogonal polarizer. One such configuration was shown in Scheme 1. In this case the reference we used BSGK solution, which can be expected to display similar temperature-, time-, or illumination-dependent changes as the sample. To optimize the sensor response the reference intensity was about 65% of the sample response, as calculated for expected a two- to threefold intensity change (Fig. 1). This reference is observed through a horizontally oriented polarizer. The sample contains BSGK, ONPG, and various concentrations of glucose and is observed through a vertically oriented polarizer. The emission from both sides of the sensor is then observed through a vertically and horizontally oriented polarizer in order to measure polarization of the system. Figure 5 shows the observed polarization of the system for BSGK + ONPG and different glucose concentrations. An advantage of polarization measurements for sensing is that they are self-normalized and thus independent of the overall intensity of the sensor.

DISCUSSION

The results shown above demonstrate that a thermostable glucokinase can serve as a glucose sensor. Additional studies are needed to obtain a BSGK-based sensor which displays larger spectral changes. For example, we are hopeful that BSGK labeled with fluorophores other than IA-ANS will display larger intensity changes, spectral shifts, or changes in lifetime. The results in the competitive RET are especially interesting because RET is a through-space interaction which occurs whenever the donor and acceptor are within the Forster distance (R_0) and does not require a conformational change and/or a change in the probe environment. For these reasons we are confident that BSGK can be used with longer wavelength donors and acceptors to develop practical glucose sensors for use in diabetes health care. Since the measurements through the skin can be easily performed by using a red laser diode or a LED as an excitation source, one may envision a polarization-based device with an external calibrated standard (66) that will allow noninvasive glucose determinations. The main advantage of using this method is the obtainment of ratiometric polarization measurements that are not influenced by light instability and sample perturbation.

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SCHEME 1.
Polarization sensing with a reference solution.

FIG. 1. Polarization sensing. Simulations of the expected changes in polarization for different values of k . For details see Materials and Methods.

FIG. 2.

Stability of BSGK and YHX at room temperature. Enzymatic measurements were performed as described under Materials and Methods. Fluorescence measurements were performed at room temperature. Ex = 290 nm; Em = 340 nm. A NATA solution was used as reference.

FIG. 3.
o-Nitrophenyl- β -D-glucopyranoside (ONPG).

FIG. 4.

Effect of glucose on the intensity emission of BSGK in the presence of $30 \mu\text{M}$. The excitation was at 290 nm, and the emission was recorded at 340 nm. $[\text{BSGK}] = 3 \mu\text{M}$.

FIG. 5.

Effect of glucose on the polarization spectra of BSGK in the presence of 30 μM ONPG.

Excitation was at 290 nm. [BSGK] = 3 μM .