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Protein-Based Biosensors for Diabetic Patients

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In this article we show the recent progress in the field of glucose sensing based on the utilization of enzymes and proteins as probes for stable and non-consuming fluorescence biosensors. We developed a new methodology for glucose sensing using inactive forms of enzymes such as the glucose oxidase from *Aspergillus niger*, the glucose dehydrogenase from the thermophilic microorganism *Thermoplasma acidophilum*, and the glucokinase from the thermophilic eubacterium *Bacillus stearothermophilus*. Glucose oxidase was rendered inactive by removal of the FAD cofactor. The resulting apo-glucose oxidase still binds glucose as observed from a decrease in its intrinsic tryptophan fluorescence. 8-Anilino-1-naphthalene sulfonic acid was found to bind spontaneously to apo-glucose oxidase as seen from an enhancement of the ANS fluorescence. The steady state intensity of the bound ANS decreased 25% upon binding of glucose, and the mean lifetime of the bound ANS decreased about 40%. These spectral changes occurred with a midpoint from 10 to 20 mM glucose, which is comparable to the K_D of holo-glucose oxidase. The ANS-labeled apo-glucose dehydrogenase from *Thermoplasma acidophilum* also displayed an approximate 25% decrease in emission intensity upon binding glucose. This decrease can be also used to measure the glucose concentration. The thermophilic apo-glucose dehydrogenase was also stable in the presence of organic solvents, allowing determination of glucose in the presence of acetone. The third enzyme used for glucose sensing was the glucokinase from *Bacillus stearothermophilus*. A fluorescence competitive assay for the determination of glucose was developed based on the utilization of this thermostable enzyme. Taken together, our results show that enzymes which use glucose as their substrate can be used as reversible and non-consuming glucose biosensors in the absence of required co-factors. Moreover, the possibility of using inactive apo-enzymes for a reversible sensor greatly expands the range of proteins which can be used as sensors, not only for glucose, but for a wide variety of biochemically relevant analytes.

KEY WORDS: Glucose; proteins; thermophilic organisms; fluorescence; biosensors.

INTRODUCTION

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]. Non-invasive 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 the medical needs there continues to be intensive efforts to develop sensors for glucose [4–9]. The

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absence of a suitable non-invasive glucose measurement has resulted in 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 coworkers [16–19], who developed a competitive glucose assay which does not require substrate and does not consume glucose. This assay used fluorescence resonance energy transfer (FRET) 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 FRET efficiency. The addition of glucose resulted in its competitive binding to ConA, displacement of ConA from the labeled dextran, and a decrease in the FRET efficiency. These early results generated considerable enthusiasm for fluorescence sensing of glucose [20–22]. The glucose-ConA system was also studied in the Lakowicz's lab. The researchers, however, found the system was only partially reversible upon addition of glucose, became less reversible with time, and showed aggregation [23–25]. For this reason, the use of other glucose-binding proteins as sensors was explored from several labs [26–30].

In fact, there is considerable interest in using enzymes or 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 interstitial 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 min have been suggested [42].

In the present report we describe the use of inactive form of enzyme probes for a non-consuming analyte stable biosensor, in particular we will show the data obtained on the glucose oxidase from *Aspergillus niger*, the glucose dehydrogenase from *Thermoplasma acidophilum*, and the glucokinase from *Bacillus stearothermophilius*.

GLUCOSE OXIDASE FROM *Aspergillus niger*

Glucose oxidase (GO) (EC 1.1.3.4) from *Aspergillus niger* catalyzes the conversion of β-D-glucose and oxygen to D-glucono-1,5-lactone and hydrogen peroxide. It is a flavoprotein, highly specific for β-D-glucose, and is widely used to estimate glucose concentration in blood or urine samples through the formation of colored dyes [43]. Because glucose is consumed, this enzyme cannot be used as a reversible sensor. In a recent report [44] we extended the use of GO under conditions where no reaction occurs. In particular, in order to prevent glucose oxidation, we removed the FAD cofactor that is required for the reaction.

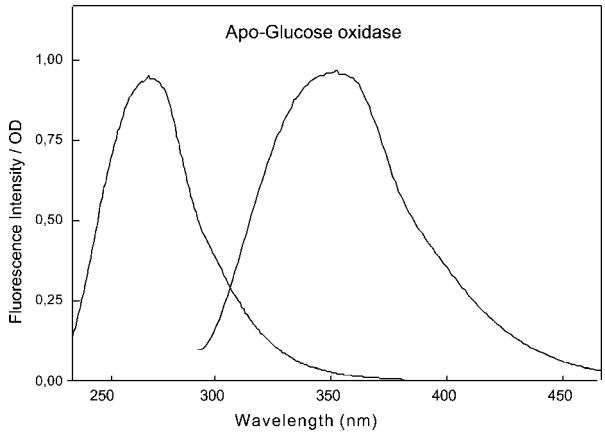

Fig. 1. Absorption and emission spectra of apo-glucose oxidase.

Figure 1 shows the absorption and emission spectra of apo-GO. The absorbance spectrum shows the characteristic shape of the coenzyme-free proteins, with a maximum of absorbance at 278 nm due to the aromatic amino acid residues. The absence of absorption at wavelengths above 300 nm indicates the FAD has been completely removed. The fluorescence emission spectrum of apo-GO at room temperature upon excitation at 298 nm displays an emission maximum at 340 nm, which is characteristic of partially shielded tryptophan residues. The addition of 20 mM glucose to the enzyme solution resulted in a quenching of the tryptophanyl fluorescence emission about 18%. This result indicates that the apo-GO is still able to bind glucose. The observed fluorescence quenching may be mainly ascribed to the tryptophanyl residue 426. In fact, as shown by X-ray analysis (Fig. 2) and molecular dynamics simulations, the glucose-binding site of GO is formed by Asp 584, Tyr 515, His 559 and His 516. Moreover, Phe 414, Trp 426 and Asn 514 are in locations where they might form additional contacts to the glucose.

The intrinsic fluorescence from proteins is usually not useful for clinical sensing because of the need for complex or bulky light sources and the presence of numerous proteins in most biological samples. ANS is known to be a polarity-sensitive fluorophore which displays an increased quantum yield in low polarity environments [44].

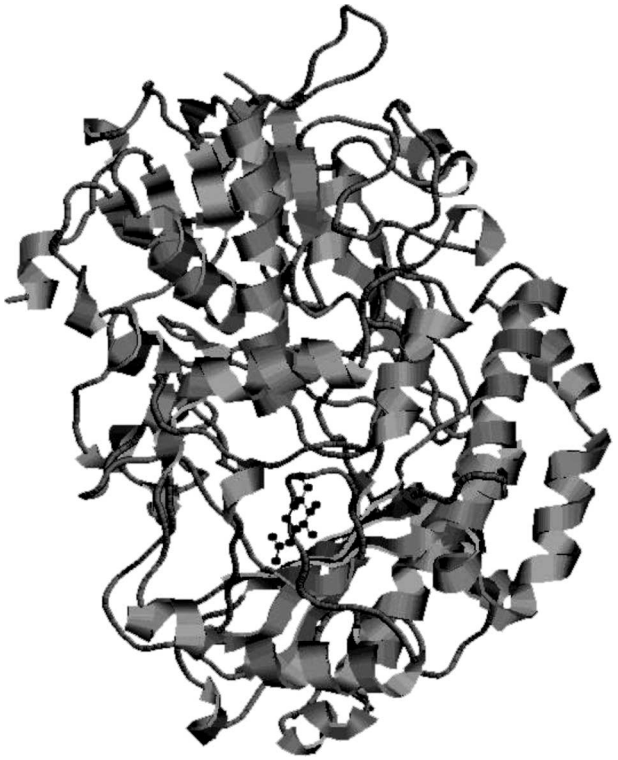

Fig. 2. Structure of glucose oxidase as solved by X-ray.

Additionally, ANS frequently binds to proteins with an increase in intensity. We examined the effects of GO on the emission intensity of ANS. Addition of apo-GO to an ANS solution resulted in an approximate 30-fold increase in the ANS intensity. Importantly, the intensity of the ANS emission was sensitive to glucose, decreasing approximately 25% upon glucose addition (Fig. 3). The ANS was not covalently bound to the protein. Addition of glucose resulted in a progressive decrease in the ANS fluorescence intensity. This suggests that the ANS is being displaced into a more polar environment upon binding glucose. The decreased ANS intensity occurred with a glucose-binding constant near 10 mM, which is comparable to the K_D of the holoenzyme. Since the binding affinity has not changed significantly, one can suggest that the binding is still specific for glucose.

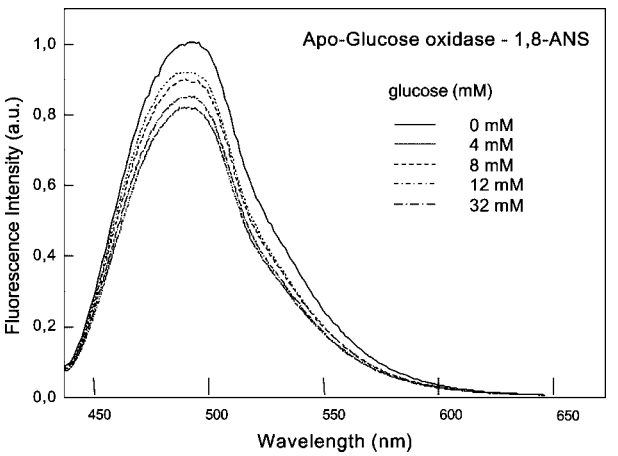

Fig. 3. Emission spectra 1,8-ANS-glucose oxidase in the presence of different amount of glucose.

Intensity decays of ANS-labeled apo-GO in the presence of glucose were studied by frequency-domain fluorometer. Addition of glucose shifts the frequency responses to higher frequencies, which is due to a decreased ANS lifetime (data not shown). The shorter lifetimes of ANS apo-GO in the presence of glucose is consistent with the suggestion that glucose displaces the ANS to a more polar environment [27].

The mean lifetime decreases by over 40% upon addition of glucose. These results demonstrate that apoglucose oxidase, when labeled with suitable fluorophores, can serve as a protein sensor for glucose.

THERMOSTABLE GLUCOSE DEHYDROGENASE FROM *Thermoplasma acidophilum*

The widespread use of proteins as sensors depends on protocols to enhance their stability, such as introduction of changes in the amino acid composition leading to enhanced protein structural stability [45]. An alternative method is to use naturally thermostable enzymes or proteins isolated from thermophilic microorganisms: These macromolecules have intrinsically stabile structural features [46,47] and they can be considered as ideal biosensors.

Glucose dehydrogenase (GD) from the thermoacidophilic archaeon *Thermoplasma acidophilum* is a tetramer of about 160 kDa composed of four similar subunits of about 40 kDa each. The enzyme shows a K_D value of 10 mM for glucose, and it is resistant to high temperatures and organic solvents. At 55°C, full activity is retained after 9 hr, and at 75°C the half-life is approximately 3 hr. Moreover, incubation of the enzyme for up 6 hr at room temperature with 50% (v/v) methanol, acetone or ethanol without any appreciable loss of activity [45]. We examined the potential of this thermostable GD as a glucose sensor. The enzyme catalyzes the following reaction:

$$\text{Glucose} + \text{NAD(P)}^+ 6\ \text{gluconate} + \text{NAD(P)}^+ + \text{H}^+$$

To prevent the glucose oxidation we used the apo-form of GD, that is the enzyme without the cofactor which

is required for the reaction. We found that the apo-GD still binds glucose with an affinity comparable to the holo-enzyme.

However, GD from *Thermoplasma acidophilum* is a thermophilic protein and can be expected to be rigid under mesophilic conditions. We knew that thermophilic proteins often display increased activity at higher temperatures or the presence of non-polar solvents [48], which are conditions expected to increase the protein dynamics. Addition of acetone to the solution containing ANS and GD resulted in a dramatic increase in the ANS intensity as well as in a blue-shift of the emission maximum. Addition of similar amounts of acetone to ANS in the absence of the protein produced modest fluorescence increase. Hence the increase in the ANS intensity reflects a change in the local protein environment which is due to acetone. To be useful as a glucose sensor the ANS labeled GD must display usefully large spectral changes in the presence of glucose. Addition of glucose to ANS-GD in the presence of 3% acetone resulted in an approximate 25% decrease in intensity (Fig. 4).

This seemed to be the optimal acetone concentration because smaller spectral changes were seen at lower and higher acetone concentrations (data not shown). Apparently at higher acetone concentrations the ANS is already in an environment which results in much of the possible increase in quantum yield. At lower acetone concentrations the environment surrounding the ANS changes in response to glucose in a manner which increases the ANS intensity.

In previous reports we described the value of fluorescence lifetimes as a basis for chemical sensing [49]. Hence we questioned whether the glucose-dependent decrease in intensity would be accompanied by a similar change in the ANS decay time. Glucose induces a modest shift in the response to higher frequencies, which indicate a decrease in the mean decay time. In the presence and absence of glucose the multi-exponential analysis (not shown) indicates that the decay is dominated by a subnanosecond component whose contribution is increased by glucose. However the changes in the intensity decay, or equivalent the phase and modulation, are not adequate for lifetime-based sensing. In the preceding discussion we interpreted the results in terms of a change in the protein environment caused by glucose. However, it is also possible that the changes are due to a difference in the amount of protein-bound ANS due to glucose. These preliminary data are not adequate to distinguish between these possibilities.

In previous reports we described the use of polarization sensing for systems which display changes in intensity, but not lifetime, in response to analytes [50]. Because the small intensity changes of ANS-GD in response to glucose are modest, it is important to carefully select the best conditions.

Figure 5 shows the emission polarized spectra of ANS-GD at various concentrations of glucose. The polarization decreases at higher glucose concentrations because the emission from this solution is observed through the horizontal polarizer. Moreover, the change in polarization is larger at shorter wavelengths, and this is due to the differences in the emission spectra of reference (ANS in buffer) and sample (ANS + GD) The wavelength dependent changes in polarization were used to create a calibration curve for glucose (data not shown). This curve shows that the present ANS-GD system can yield glucose concentrations accurate to about 2.5 mM, at a glucose concentration near 20 mM.

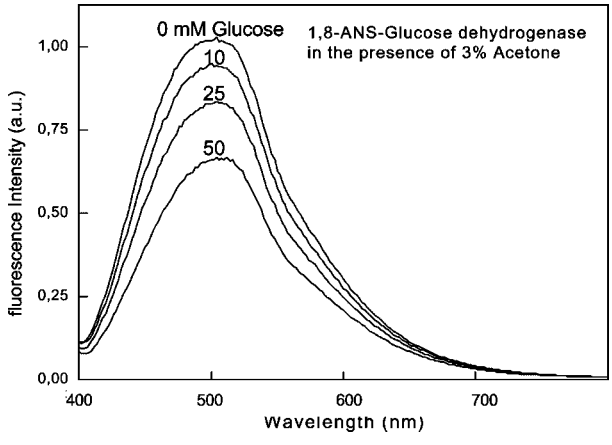

Fig. 4. Effect of glucose on the emission spectra of 1,8-ANS-glucose dehydrogenase in the presence of 3% acetone.

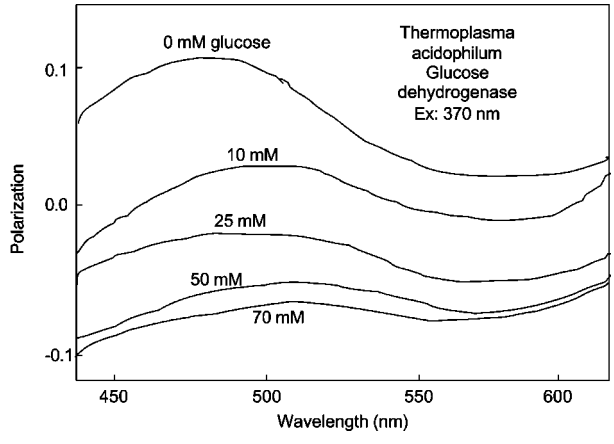

Fig. 5. Emission polarization spectra of 1,8-ANS-glucose dehydrogenase in the presence of different amount of glucose.

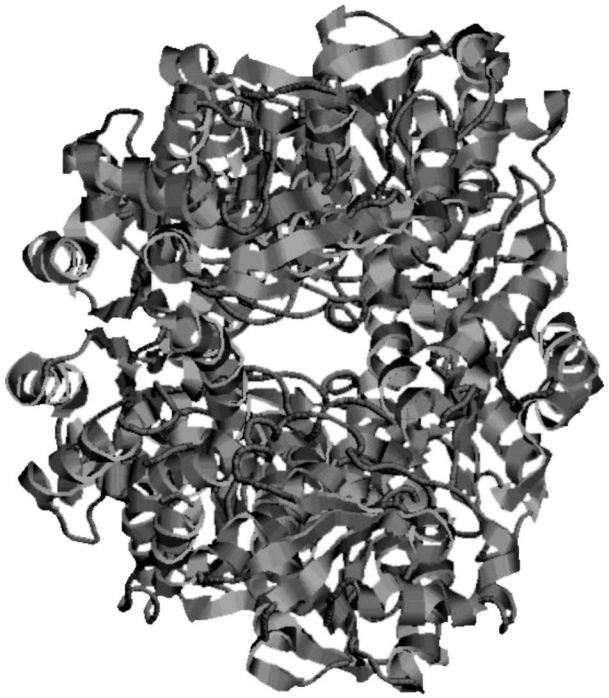

Fig. 6. Structure of the hexokinase as solved by X-ray.

A FLUORESCENCE COMPETITIVE ASSAY BY USING THE STABLE GLUCOKINASE

The structure of the hexokinase A from yeast has been determined by X-ray diffraction both in the absence and presence of glucose (Fig. 6) [51,52]. The polypeptide chain of 485 amino 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 [53–55]. 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 [53–55].

For use as a sensor a protein should have long term stability. Unfortunately, yeast hexokinase and human hexokinase are unstable and quickly loose activity at room temperature. Thermophilic micro-organisms produce enzymes with unique characteristics such as high temperature-, chemical-, and pH-stability. These enzymes are already in use as bio-catalysts in industrial processes [45]. A glucokinase from the thermophilic organism *Bacillus stearothermophilus* has been characterized and is known to display long term stability [56]. Hence we evaluated the use of this glucokinase (BSGK) in the absence of ATP as a glucose non-consuming glucose sensor. This protein has already been used as an active enzyme in glucose assays [56].

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 7 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 two weeks at room temperature and the fluorescence intensity remains constant. Hence BSGK is a good candidate for a glucose sensing probe.

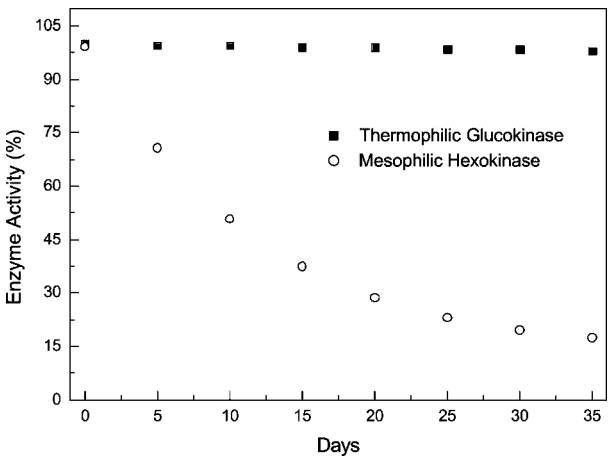

Fig. 7. Stability of the thermostable glucokinase. The incubation was at room temperature.

BSGK has a single cysteine residue located near the active site [57]. We labeled the residue with a sulfhydral-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 conclusions from these observations is that BSGK binds glucose in the absence of ATP and can thus serve as a non-consuming 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.

Resonance energy transfer (RET) reliably occurs whenever fluorescent donors and acceptors are in close proximity. 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. Addition of ONPG (3 μM) 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 [58]. 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 [58].

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. 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 2–3-fold intensity change. This reference is observed through a horizontally oriented polarizer [58]. The sample contains

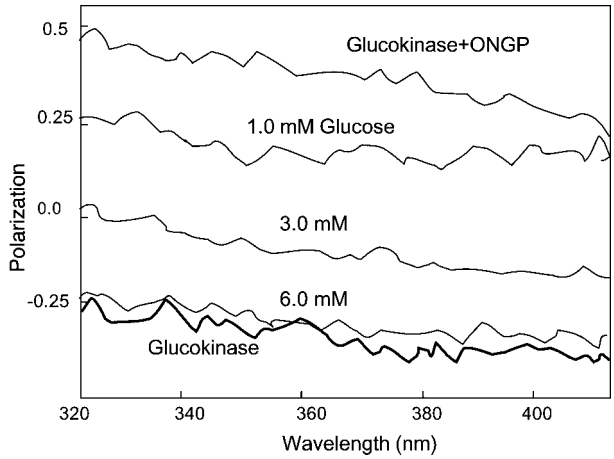

Fig. 8. Effect of glucose on the emission polarization spectra of the thermostable glucokinase in the presence of ONGP.

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 8 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.

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 light emitting diode (LED) as an excitation source, one may envision a polarization based device with an external calibrated standard 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.

CONCLUSIONS

The results described represent our first attempt to use inactive forms of enzymes as glucose sensor. Larger glucose-dependent spectral change would increase the accuracy of the glucose measurements. In spite of these difficulties we feel that our system demonstrates a useful approach to sensing. Our results suggest that the enzymes which use glucose as their substrate can be used as reversible and non-consuming glucose sensors in the absence of required co-factors. The possibility of using inactive apo-enzymes for a reversible sensor greatly expands the range of proteins which can be used as sensors, not only for glucose, but for a wide variety of biochemically relevant analytes. Hence one is no longer limited to using signaling proteins which bind the analyte without chemical reaction. The need for acetone may be eliminated by selecting proteins which are less thermophilic. The proteins can be engineered for covalent labeling by insertion of cysteine residues at appropriate locations in the sequence. The glucose induced spectral changes may be larger with other polarity sensitive probes or by the use of RET between two fluorophores on the protein. In summary, apo-enzymes appear to be a valuable source of protein sensors.

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