

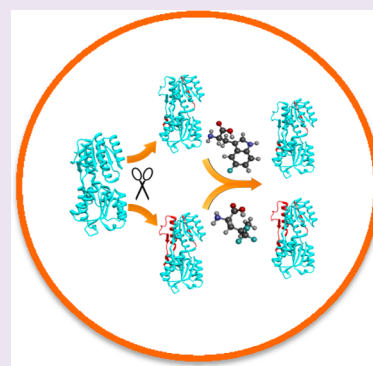
Glucose Recognition Proteins for Glucose Sensing at Physiological Concentrations and Temperatures

Smita Joel, Kendrick B. Turner, and Sylvia Daunert*

Department of Biochemistry and Molecular Biology, Miller School of Medicine, University of Miami, 1011 NW 15th Street, Miami, Florida 33136, United States

Supporting Information

ABSTRACT: Advancements in biotechnology have allowed for the preparation of designer proteins with a wide spectrum of unprecedented chemical and physical properties. A variety of chemical and genetic methods can be employed to tailor the protein's properties, including its stability and various functions. Herein, we demonstrate the production of semisynthetic glucose recognition proteins (GRPs) prepared by truncating galactose/glucose binding protein (GBP) of *E. coli* and expanding the genetic code via global incorporation of unnatural amino acids into the structure of GBP and its fragments. The unnatural amino acids 5,5,5-trifluoroleucine (FL) and 5-fluorotryptophan (FW) were chosen for incorporation into the proteins. The resulting semisynthetic GRPs exhibit enhanced thermal stability and increased detection range of glucose without compromising its binding ability. These modifications enabled the utilization of the protein for the detection of glucose within physiological concentrations (mM) and temperatures ranging from hypothermia to hyperthermia. This ability to endow proteins such as GBP with improved stability and properties is critical in designing the next generation of tailor-made biosensing proteins for continuous *in vivo* glucose monitoring.



Reliable, low cost technologies for glucose sensing has been the focus of a continuous active field of research since the first glucose biosensing device was proposed in 1962.^{1,2} Despite recent technological improvements in consumer devices, current-generation commercially available glucose meters still measure glucose by employing electrochemical detection based on traditional enzymes, such as glucose oxidase (GOx) or glucose-1-dehydrogenase (GDH).¹ These electrochemical methods exhibit poor performance in the hypoglycemic range and suffer from hematocrit dependence and interference from electrochemically active molecules, hypoxemia, or hypotension.¹ Lack of selectivity is especially troublesome, as the list of interfering compounds includes molecules commonly found in blood such as acetaminophen, salicylic acid, ibuprofen, ascorbic acid, etc.² To overcome these limitations, alternative technologies based on an optical response using rationally engineered glucose sensing proteins are being explored. To that end, research is being aimed at achieving reagentless optical sensing systems that are sensitive, selective, reproducible, accurate, rugged, and capable of glucose detection at physiological concentrations and temperatures. Among new methods developed are those based on fluorescence,^{3–5} fluorescence resonance energy transfer (FRET),^{3,5} and bioluminescence.⁶ These optical methods exploit the hinge-motion conformational change exhibited by the glucose/galactose binding protein (GBP) (Figure 1), a periplasmic binding protein found in many bacteria that undergoes a conformational change upon binding glucose. GBP has been extensively studied as a possible sensing component of future generations

of devices for the continuous, real-time monitoring of glucose for the management of diabetes.^{3,4,6,7}

The dissociation constant (K_D) of wild-type GBP is 0.2 μM ,⁴ which is too low to be useful at physiologically relevant glucose concentrations that range from 2 to 20 mM. This has constrained GBP's widespread incorporation into commercial devices. Efforts to alter the binding affinity of proteins through changes in the amino acid sequence have included site-directed mutagenesis,^{3,7–9} random mutagenesis,⁷ and DNA shuffling.¹⁰ Amiss et al. generated a library by random mutation of amino acids in the binding pocket of GBP. On screening the library, the single mutation A213R was found to alter the GBP glucose affinity to 1 mM.⁷ In another examples, triple and double mutants of GBP obtained by site-directed mutagenesis, H152C/A213R/L238S³ and D14E/F16A,⁸ respectively, demonstrated higher K_D 's. These efforts have resulted in proteins with altered affinities, but the selectivity toward glucose against other sugars and glycosylated moieties has not been fully explored. Since these approaches introduce significant changes into the protein binding pocket, they may adversely affect the selectivity of the protein.

While previous work to successfully achieve physiological binding affinities has been performed by employing site directed mutagenesis approaches, herein we undertake a different strategy, namely, truncation of the original protein

Received: September 5, 2013

Accepted: May 19, 2014

Published: May 19, 2014

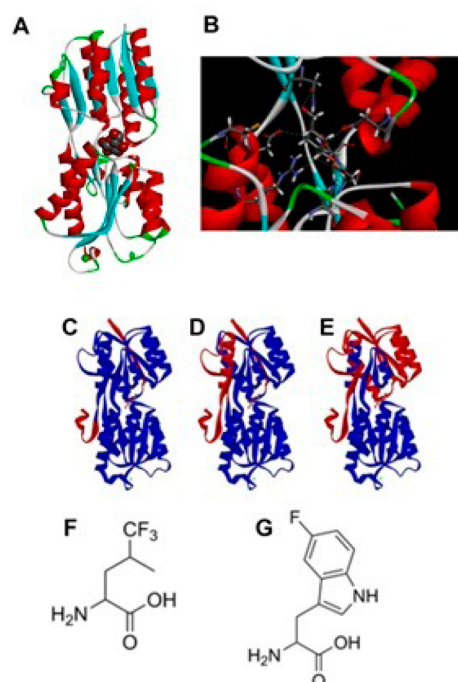


Figure 1. Structures of the protein and the unnatural amino acid analogues incorporated into the proteins. (A) Native GBP with glucose in the ligand-binding pocket and Ca^{2+} present in the Ca^{2+} -binding pocket (PDB ID: 2GBP). (B) GBP's binding pocket is magnified, showing Cys152 and the amino acids involved in H-bonding with glucose. (C) tGRP1- (D), tGRP2-, and (E) tGRP3-truncated areas of the original GBP protein are shown in red. (F) 5,5,5-Trifluoroleucine. (G) 5-Fluorotryptophan.

to achieve the same goal. Truncation provides the advantage of not disrupting the first layer of recognition of the protein, thus keeping intact the original binding pocket, in contrast to site-directed mutagenesis, which introduces a different amino acid. In some proteins, mutagenesis causes total loss of recognition, while in others it is partial, giving rise to proteins with varied degrees of recognition. Since truncation does not interfere with the first layer of molecular recognition, changes in binding ability can be better controlled, and therefore, such a strategy may be valuable for retention of the molecular recognition between the protein and its ligand.

To that end, we hypothesized that, by truncating GBP H152C (full length GBP with a cysteine at position 152) and incorporating fluorinated unnatural amino acids into these fragments as well as into full length GBP H152C, we could construct glucose recognition peptides (truncated GBP H152C, tGRPs, and unnatural amino acid incorporated peptides, uGRPs) with altered dissociation constants while maintaining glucose response and improving thermal stability. Specifically, three tGRPs consisting of amino acids 14–296, 14–256, and 87–271 were designed (Figure 1). Using these tGRPs and full length GBP, we globally incorporated the unnatural amino acids 5-fluorotryptophan (FW) and 5,5,5-trifluoroleucine (FL) to replace natural leucines and tryptophans throughout the protein structure (Figure 1). The binding characteristics and thermal stabilities of these engineered proteins were evaluated. Care was taken to maintain much of the hydrogen-bonding network that interacts with glucose, as well as the hinge region connecting the two lobes so that glucose binding is preserved.

Herein, we present a series of newly designed tGRPs and uGRPs, their characterization, and their use in glucose sensing.

RESULTS AND DISCUSSION

Truncation of Full Length GBP. By truncating the structure of GBP H152C, we hypothesized that the resulting perturbation in the structure of the proteins would result in an altered affinity for glucose. It has been shown that truncated proteins, like those produced by mutagenesis, can exhibit altered activity, stability, folding, and binding affinities.^{11,12} To investigate the effect of truncating GBP's affinity for glucose and thermal stability of the protein, three truncated versions of GBP H152C were engineered. The proteins were rationally designed to maintain a functional hinge region to preserve the ability to undergo a conformational change. tGRP1 included all amino acids through residue 296 in order to maintain all three strands of the hinge region of the protein structure. In the design of tGRP2, all amino acids located after residue 256 were removed, thus maintaining two of the three strands composing the hinge region. In order to maintain activity and selectivity toward glucose, the native structure was truncated such that most of the amino acids involved in hydrogen-bonding interactions of the binding site were unperturbed. Native GBP interacts with glucose through a network of hydrogen-bonding interactions involving Asp14, Asn91, His152, Asp154, Arg158, Asn211, Asp236, and Asn256 (Figure 1).¹³ These eight amino acids comprise the first-shell or layer interactions. In addition, ten more amino acids interact with the first-shell amino acids to stabilize their structure around the sugar ligand. While both tGRP1 and tGRP2 maintain all of the first-shell interacting amino acids, part of their second-shell or layer amino acids were removed. Specifically, tGRP1 and tGRP2 removes one and four second-shell amino acids, respectively. In the design of tGRP3, much of one lobe of the protein, one strand of the hinge region, and one amino acid from the binding pocket were removed, and as a result, this protein lacked any glucose-binding activity (data not shown).

Characterization of Truncated GBP Fragments, tGRPs.

The three different proteins were expressed in *E. coli* and chemically modified via site-selective labeling of a unique Cys residue with a fluorescent coumarin probe, namely, 7-diethylamino-3-(((2-maleimidyl)ethyl)amino)carbonyl coumarin (MDCC). The truncated proteins were characterized in terms of their binding ability to glucose and other sugars. Upon glucose binding, the fluorescence intensity generated by the MDCC-labeled tGRPs was decreased. Since the MDCC-modified Cys is located on a flexible region at the edge of the binding pocket, we hypothesize that when glucose binds, MDCC is displaced from the binding pocket and oriented more into the solution, thus decreasing its fluorescence. It was found that as the protein was increasingly truncated, the apparent K_D observed was increased, thus decreasing its affinity for glucose (Figure 2). As more of the stabilizing, second-shell amino acids were removed, the hydrogen-bonding interactions with glucose became disrupted, which resulted in an increased K_D of 75.2 μM for tGRP1 and 0.25 mM for tGRP2 (Supporting Information, Table 1).

Disrupting the first- and second-shell amino acids could also affect the selectivity of the proteins, causing them to respond to sugar molecules other than glucose and galactose. Given that the overall structure became less stable, it could be possible that the binding pocket became more flexible and able to accommodate other similarly shaped ligands. To investigate

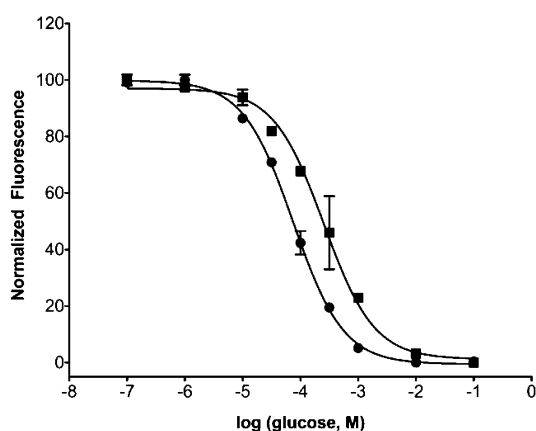


Figure 2. Normalized fluorescence response curve for tGRP1 (●) and tGRP2 (■). The glucose-response curve for tGRP1 and tGRP2 labeled at position 152 (with respect to native GBP) with MDCC. Data points represent the average of blank-subtracted triplicate samples. Error bars correspond to ± 1 SD.

this, a selectivity study with MDCC-labeled tGRPs was carried out with a variety of physiologically relevant sugar molecules (Figure 3). As with native GBP, tGRPs responded best to glucose and to a lesser degree to galactose. None of the other sugar molecules showed a significant response.

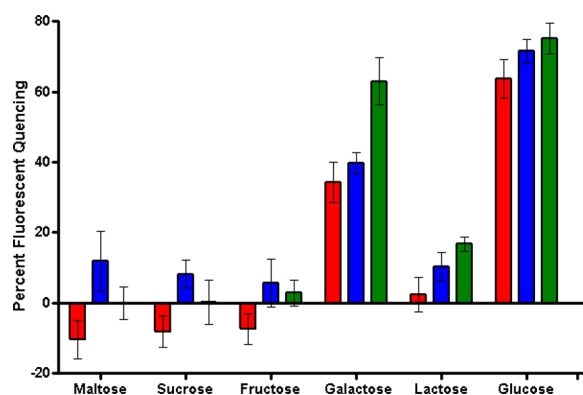


Figure 3. Response of GBP H152C (green), tGRP1 (red), and tGRP2 (blue) to different sugars (100 mM). Data are the average of ± 1 SD ($n = 3$).

To determine whether the overall structural stability of the tGRPs had been indeed affected, the thermal stability of each tGRP and GBP H152C was determined by circular dichroism (CD) spectroscopy. Protein thermal stability is an important consideration when developing protein-based sensors that will be used for extended periods of time at 37 °C, the temperature of the human body. Improved thermal stability should increase the lifetime of the sensor, allowing for long-term, reproducible glucose determination. GBP H152C has a melting temperature (T_m) of 52.4 °C (Figure 4a). Truncating the native structure had a significant effect on the thermal stability. A decrease in the T_m was observed as the size of the protein decreased after truncation (Figure 4). Since tGRP3 showed no glucose response, it was not further characterized. We hypothesized that this drastic loss in thermal stability reflects the instability of the protein structure as a result of truncation, which likely contributes to the increase in dissociation constants for its ligand, glucose.

Further characterization of the tGRPs by circular dichroism revealed that, similar to native GBP, there was little change in the overall secondary structure characteristics of the proteins. This is evident from the far-UV CD absorbance spectra for GBP152, tGRP1, and tGRP2 shown in Figure 5a. The α -helix peak (approximately 210 and 220 nm) for GBP H152C is much intense than that of tGRPs showing a loss of α -helical structure on truncation.

Global Incorporation of Unnatural Amino Acids in tGRPs. Incorporation of unnatural amino acids into a protein structure results in changes of protein properties, function, and stability.^{14–18} Several fluorinated unnatural amino acids such as fluorovaline,¹⁹ fluoroleucine,^{17,19,20} fluoroisoleucine,²¹ fluorophenylalanine,²² and fluoroproline²³ have been shown to enhance thermal, conformational, and chemical stability. Fluorine has similar van der Waals radii as hydrogen and, therefore, can be replaced for hydrogen in amino acids with minimal steric perturbation of the protein.²⁴ Improved thermal stability can contribute to increased shelf-lives, longer continuous use capabilities, decrease in refrigeration storing needs, easier packaging/transport of these peptides, and use at ambient, physiological, and extreme environments.

Full length GBP H152C counts with 22 leucines and 5 tryptophans, tGRP1 with 5 tryptophans and 21 leucines, and tGRP2 with 4 tryptophans and 19 leucines. Since one tryptophan is located within the binding pocket, expanding the natural genetic code by incorporation of FWs in the protein might affect the binding ability and/or stability of the protein.²⁵ Fluoroleucines have been shown to enhance the thermal stability of proteins. Therefore, we decided to evaluate our hypothesis that global incorporation of FL and FW in tGRP1 and tGRP2 may result in changes in the structure and/or alter the stability of the proteins. Global incorporation of unnatural amino acids was carried out as outlined in Methods. To confirm the incorporation of unnatural amino acids, the purified proteins were analyzed by LC–ESI-MS/MS. The difference in protein mass obtained before and after unnatural amino acid incorporation confirmed the incorporation of the unnatural amino acids (Supporting Information, Table 2).

Characterization of the Unnatural Amino Acid Incorporated tGRPs, uGRPs. The glucose binding ability of the uGRPs was evaluated. Dose–response curves for glucose generated with the uGRPs with FW and FL, respectively, are shown in Figure 6. The percent fluorescence quenching of uGRPs labeled with MDCC in solution containing 10^{-3} M glucose is shown in Supporting Information, Table 3. Glucose binding was maintained with the GRPs containing unnatural amino acids. Apparent K_D 's were determined to be 2.02×10^{-6} M, 4.1×10^{-6} M, 7.5×10^{-4} M, 5.4×10^{-5} M, 1.9×10^{-4} M and 1.8×10^{-4} M for uGRP-FW, uGRP1-FW, uGRP2-FW, uGRP-FL, uGRP1-FL and uGRP2-FL, respectively (Supporting Information, Table 1). It was observed that proteins with unnatural tryptophan demonstrated lower detection limits compared to the proteins with unnatural leucines. In order to further characterize the structural changes resulting from unnatural amino acid incorporation and the corresponding effects on binding, far-UV CD analysis was carried out. The far-UV CD spectrum of uGRP-FW was similar to the CD spectrum of GBP152, indicating that the secondary structure of GBP was not significantly altered by incorporation of unnatural tryptophans (Figure 5b). This was further supported by the apparent K_D 's of both GBP H152C⁴ and uGRP-FW in the micromolar range. However, the CD spectrum of uGRP-FL

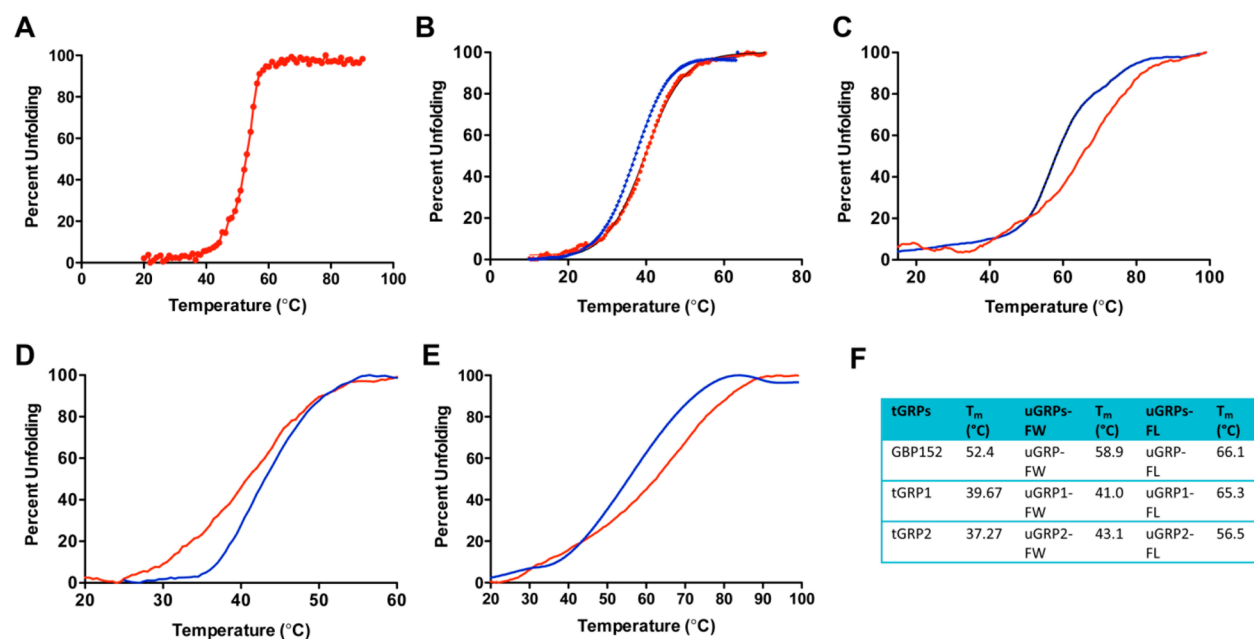


Figure 4. Thermal denaturation curves for (A) GBP H152C, (B) tGRP1 (red) and tGRP2 (blue), (C) uGRPFL (red) and uGRPFW (blue), (D) uGRP1FW (red) and uGRP2FW (blue), (E) uGRP1FL (red) and uGRP2FL (blue). (F) Table showing the melting temperatures of proteins. Proteins were prepared at a concentration of 0.2 mg mL^{-1} in buffer (10 mM phosphate, 0.2 mM CaCl_2 , pH = 7.5). Alpha helix denaturation was monitored by CD at 222 nm as the temperature was increased from 10 to 70 °C. All T_m values are the average of ± 0.1 to ± 0.6 SD ($n = 2$).

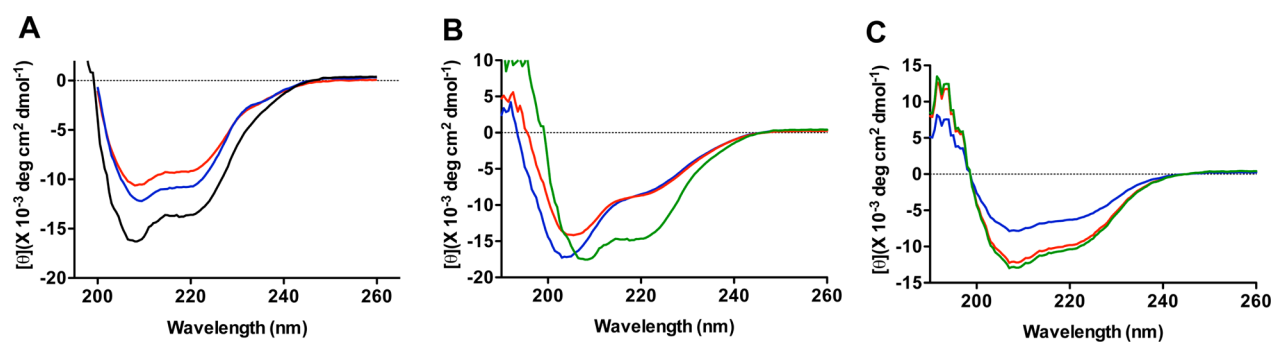


Figure 5. Far-UV CD absorbance of (A) GBP H152C (black), tGRP1 (red), and tGRP2 (blue). (B) uGRPFW (green), uGRP1FW (red), and uGRP2FW (blue). (C) uGRPFL (green), uGRP1FL (red), and uGRP2FL (blue). Three accumulations were averaged for each sample at RT. The response for each blank was subtracted from the response for the corresponding sample, and the resulting spectra are shown.

(Figure 5c) was different from that of GBP152, as the peaks at 222 and 208 nm were less intense for uGRP-FL. This suggested that the secondary structure of GBP152 was altered by incorporating unnatural leucines. This may explain the change in apparent K_D as compared to GBP H152C. The CD spectrum of uGRP1/2-FW (Figure 5b) revealed that the incorporation of fluorinated tryptophans in the truncated proteins, i.e., tGRP1/2, altered the secondary structure of the protein as compared to the full length GBP-FW (uGRP-FW). This was explained again by both the position and intensity of peaks at 222 and 208 nm. The peak at 222 nm for tGRP1/2-FW was less pronounced than the corresponding peak for uGRP-FW, while the peak at 208 nm in uGRP-FW was shifted toward lower wavelength (204 nm) in tGRP1/2-FW. Also, when comparing the secondary structure of tGRP1/2-FW with that of tGRP1/2, it was evident that the peak around 208 nm, as seen in both the tGRP1/2, is shifted to a lower wavelength of 204 nm in tGRP1/2-FW, suggesting a decrease in alpha helical content. However, the CD spectrum of tGRP1/2-FL (Figure 5c) suggests that the incorporation of fluorinated leucines in

tGRP1/2 alters the secondary structure when compared to tGRP1/2, as evident from the difference in the intensities of peaks.

The thermal stability (T_m) of the uGRPs-FW and uGRP-FL was determined by CD spectroscopy (Figure 4c–e). Incorporation of FW and FL into both uGRPs resulted in enhanced thermal stability when compared to the full length GBP and tGRPs. Incorporation of fluorinated leucines demonstrated a larger increase in melting temperature when compared to fluorinated tryptophans. It was also observed that increase in T_m can be correlated to the number of leucines/tryptophans in the protein. tGRP1 has 21 leucines, and tGRP2 has 19 leucines. uGRP2-FL, which has a total of 6 fluorines less than uGRP1-FL, has a T_m that is 10 °C lower than that of uGRP1-FL. However, uGRP1/2-FW have similar T_m , which can be explained by the fact that there is a difference of only one fluorine in the structure of the two proteins. Thus, global incorporation of FW and FL resulted in an increased T_m of tGRPs.

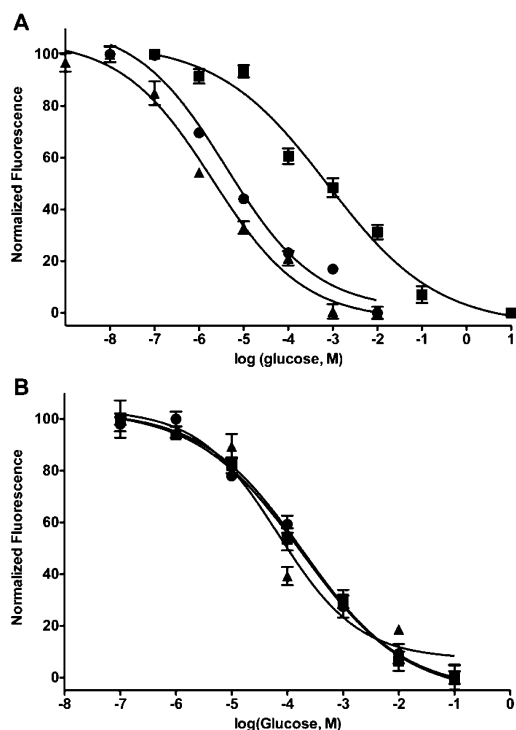


Figure 6. Normalized fluorescence response curve for uGRPs with (A) FW and (B) FL. Glucose-response curves with globally incorporated FW and FL labeled at position 152 (with respect to native GBP) with MDCC for uGRP (\blacktriangle), uGRP1 (\bullet), and uGRP2 (\blacksquare). Data points represent the average of blank-subtracted triplicate samples. Error bars correspond to ± 1 SD.

To demonstrate the ability of our genetically engineered uGRPs in sensing applications, we developed a catheter-based biosensor for the continuous detection of glucose. The fluorescence-based biosensor was developed by covalent immobilization of the MDCC-labeled uGRPs within the UV polymerized acrylamide hydrogel on the tip of an optical fiber. The response of the fiber optic glucose biosensor was evaluated in standard glucose solutions, in human serum, and in pig blood (Supplementary information, Figure 1), by monitoring the changes in fluorescence intensity of the probe. The response of the sensor was also studied at different temperatures to reflect physiological situations ranging from hypothermia to hyperthermia. The performance of the uGRP1-FW and uGRP1-FL biosensors in glucose solutions in buffer was also evaluated at 37 and 42.5 °C (Figure 7). The results obtained are those that we would expect given the demonstrated thermal stability of the uGRP 1s-FW/FL. The uGRP1-FW has a T_m of 41.0 °C, and the sensors incorporating this protein showed very little change in fluorescence signal in the presence of different glucose concentrations at 42.5 °C, thus indicating the loss in activity of the protein at physiological hyperthermia. In contrast, the uGRP1-FL which has a T_m at 65.3 °C, showed significant fluorescence quenching at both 37 and 42.5 °C. Further, the hydrogel plays an important role in sensing glucose within the millimolar detection range. This can be observed for some of the unnatural amino acids incorporated GRPs (Supporting Information Table 1) whereby although the binding constant is in the micromolar range, the protein still detects glucose in the millimolar ranges (Figure 7). This is because the hydrogel functions as a barrier, allowing the diffusion of glucose to the immobilized protein in the bulk of

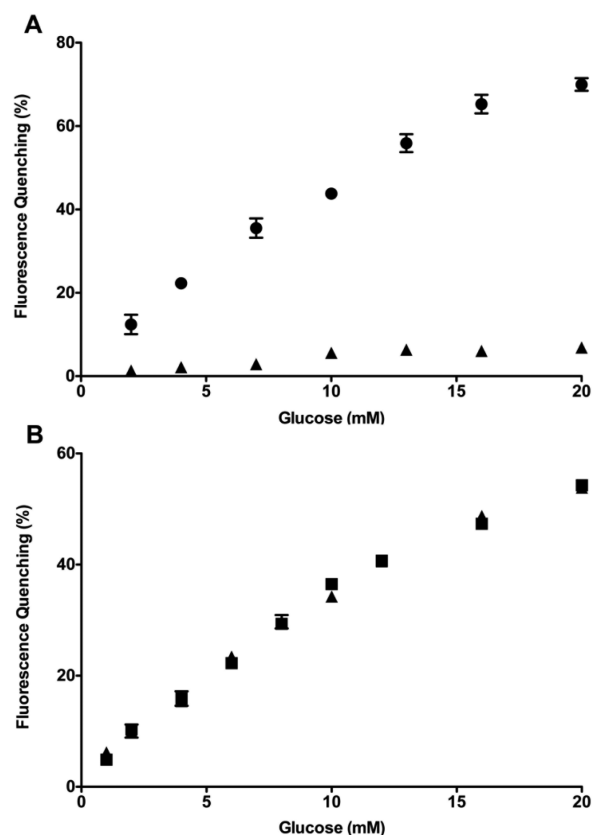


Figure 7. Temperature study of uGRP fiber optic biosensor (A) GRP1-FW and (B) GRP1-FL. Symbols represent (\bullet) 37 °C and (\blacktriangle) 42.5 °C. Data are the average of ± 1 SD ($n = 3$). Relative standard deviations at all concentrations are less than 10%.

hydrogel, thus altering the detection range and causing the sensor to detect higher levels of glucose, in this case the targeted physiological millimolar concentrations. Thus, the sensors incorporating designer proteins with enhanced thermal stability retain their binding ability toward glucose and can be employed for monitoring glucose at physiological temperatures, from hypothermia to hyperthermia. The response of a hydrogel sensor was also studied over a period of 3 days (Supporting Information, Figure 2). The hydrogel was deposited on the tip of the fiber, and the response of the sensor to glucose was measured each day. The sensor was stored at 4 °C in buffer in between measurements.

In conclusion, herein we present genetically engineered sensing proteins that can function as biosensors with desired properties based upon truncated forms of GBP from *E. coli* and expansion of the genetic code by incorporation of unnatural amino acids into these proteins. Previous work has shown wild-type GBP to have a binding constant in the micromolar range, which is not ideally suited for the development of a glucose biosensing system at physiologically relevant millimolar concentrations. The apparent binding constant of the truncated proteins is shifted from the micromolar to millimolar range, allowing for glucose determination at physiological ranges. In order to design proteins with thermal characteristics to withstand the rigors of the human body, we globally incorporated FWs and FLs into the truncated proteins. This incorporation of FWs and FLs affects the secondary structure of the proteins when compared to native protein, but the ability to bind to glucose is not compromised. More importantly, FL and

FW enhance the thermal stability of the sensing proteins. The enhanced thermal stability and altered K_D 's of these newly prepared GRPs makes them especially suited for long-term continuous glucose sensing in a variety of platforms and devices as well as for transport and long-term storage.²⁶ Moreover, it paves the way for the design and preparation of other proteins with tailor-made characteristics for a variety of biotechnology applications.

METHODS

Reagents. All oligonucleotide primers were purchased from Operon Biotechnologies (Huntsville, AL). Phusion DNA polymerase was purchased from New England Biolabs (Ipswich, MA). 2-Amino-2-hydroxymethylpropane-1,3-diol (Tris-base), β -mercaptoethanol, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), calcium chloride (CaCl_2), magnesium sulfate, thiamine, 5-fluorotryptophan, 5,5,5-trifluoro-leucine, ampicillin sodium salt, tetracycline, ethidium bromide, agarose, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), and D-glucose, sucrose, and lactose, D-galactose, acrylamide, 2,2-diethoxyacetophenone, N,N' -methylenebis(acrylamide), and 3-(trimethoxysilyl)propyl methacrylate (MPTS) were obtained from Sigma-Aldrich (St. Louis, MO). Glycine, sodium chloride, ethylenediaminetetraacetic acid (EDTA), Luria-Bertani (LB) broth, and agar were purchased from Fisher Scientific (Fair Lawn, NJ). M9 minimal salts was purchased from Difco (Sparks, MD). Methanol, acetic acid, sodium phosphate dibasic, sodium phosphate monobasic, glycerol, and bromophenol blue were purchased from VWR (Bridgeport, NJ). Imidazole and maltose were purchased from J.T. Baker (Phillipsburg, NJ). Isopropyl β -D-1-thiogalactopyranoside (IPTG) and dithiothreitol (DTT) were purchased from Gold Biotechnology (St. Louis, MO). Sodium dodecyl sulfate was ordered from Curtin Matheson (Houston, TX). The Bradford protein assay kit was purchased from Biorad (Hercules, CA). Ni-NTA agarose resin, QIAquick gel purification kit, QIAprep DNA isolation kit, and the pQE70 vector were purchased from Qiagen (Valencia, CA). TOP10F' cells, Tris-Glycine SDS PAGE gels, 7-diethylamino-3-(((2-maleimidyl)ethyl)amino)carbonyl)coumarin (MDCC), Mark12 protein standard, and 1 kb DNA standard were purchased from Invitrogen (Carlsbad, CA), and 3500 MWCO Slide-A-Lyzer 3–12 mL dialysis cassettes were purchased from Pierce (Rockford, IL). T4 DNA ligase, SphI restriction enzyme, and BglII restriction enzyme were purchased from Promega (Madison, WI). Optical fibers with core diameter of 200 μm were obtained from Ocean Optics Inc. (Dunedin, FL). UVGL-58 hand-held UV lamp for polymerization of hydrogel was obtained from UVP (Upland, CA).

Apparatus. Polymerase chain reactions (PCR) were performed using an Eppendorf Mastercycler Personal Thermocycler (Hauppauge, NY). Electrophoresis of DNA was carried out using an FB105 Fischer Biotech Electrophoresis Power Supply (Pittsburgh, PA). DNA gels were visualized using a UV Transilluminator platform from UVP (Upland, CA). Optical density measurements were taken using a Spectronic 21D from Milton Roy (Ivy Land, PA). Cells were lysed using a 550 Sonic Dismembrator from Fisher Scientific (Pittsburgh, PA). Proteins were expressed by incubating bacteria at 37 °C on a Forma Scientific Orbital Shaker (Fairlawn, NJ). All centrifugation was carried out using a Beckman J2MI centrifuge (Palo Alto, CA). Proteins were visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Invitrogen 10–20% Tris-glycine gels in an Invitrogen X Cell Sure Lock Mini Cell (Carlsbad, CA). Fluorescence measurements were obtained using a QuantaMaster 40 Spectrofluorometer from PTI (Birmingham, NJ). Circular Dichroism measurements were taken using a Jasco J-810 Spectropolarimeter (Easton, MD). DNA sequencing was performed by the Advanced Genetic Testing Center at the University of Kentucky.

Cloning of Truncated Glucose Recognition Peptides (tGRPs). To prepare tGRPs, various segments of the full-length glucose binding protein (GBP) containing a mutation introducing a unique cysteine in place of histidine at position 152 from *Escherichia coli* (*E. coli*) were amplified by PCR and ligated into the expression

vector pQE70 containing a 6 histidine tag. Specifically, gene fragments were amplified corresponding to amino acids 14–296 (tGRP1), 14–256 (tGRP2), and 87–271 (tGRP3) of the native sequence. For the amplification of tGRP1, primers tGBP(14)/fwd [5'-GGTGGTGC-ATGCGCGATAACTTTATGTCTGTAGTGC GG-3'] and tGBP(-296)/rev [5'-GGTGGTAGATCTAACATAAGGTACGCGGACCAC-3'] were used. For the amplification of tGRP2, primers tGBP(14)/fwd and tGBP(-256)/rev [5'-GGTGGTAGATCTGTTCAGTACGGT-GCCCCCAG-3'] were used. For the amplification of tGRP3, primers tGBP(87)/fwd [5'-GGTGGTGCATGCGCGTGGTT-TTCTTCAACAAAGAACC-3'] and tGBP(-271)/rev [5'-GGTGGTAGATCTGTTTTTCGCCAGATCAAAGGTCCG-3'] were used. PCR was carried out using Phusion High-Fidelity DNA Polymerase. PCR conditions consisted of an initial denaturation period of 30 s at 98 °C. Next, 30 cycles of 98 °C for 30 s, 70 °C for 30 s, and 72 °C for 60 s followed by a final elongation period of 72 °C for 5 min was carried out. The resulting reaction products were analyzed by TAE 1% agarose gel electrophoresis, and the appropriate DNA fragments (861 bp for tGRP1, 741 bp for tGRP2, and 567 bp for tGRP3) were excised from the gel and purified using the QIAquick gel extraction kit.

The DNA fragments tGRP1, tGRP2, and tGRP3 along with expression vector pQE70 were digested with restriction enzymes BglII and SphI. The resulting products were analyzed by TAE 1% agarose gel electrophoresis, excised from the gel, and purified using the QIAquick gel extraction kit. A gel slice of digested pQE70 was co-purified in each separate tube containing tGRP1, tGRP2, and tGRP3 allowing the vector and insert to be eluted from the purification column simultaneously. To the eluted DNA were added T4 DNA ligase and T4 DNA ligase buffer (10x), and the ligation reaction was allowed to proceed overnight at RT. The ligated DNA was transformed into TOP10F' cells. Plasmid DNA was isolated from overnight cultures of selected transformants and analyzed for the presence of the desired DNA fragments by digestion with BglII and SphI. DNA sequencing was also performed to confirm the correct DNA sequence.

tGRPs Expression and Purification. Plasmids *tGRP1* and *tGRP2* were transformed into TOP10F' chemically competent cells. For protein expression, cells were grown overnight at 37 °C with shaking in 3 mL of LB broth containing ampicillin (100 $\mu\text{g mL}^{-1}$) and tetracycline (12.5 $\mu\text{g mL}^{-1}$) for selection. The following day in a 1 L flask, 500 mL of LB broth containing ampicillin (100 $\mu\text{g mL}^{-1}$) and tetracycline (12.5 $\mu\text{g mL}^{-1}$) was inoculated with the overnight culture and grown at 37 °C with shaking at 250 rpm to an OD_{600} of 0.4–0.5. Protein expression was then induced with IPTG at a final concentration of 1.0 mM. The expression culture was grown overnight at RT with shaking.

For protein purification, the culture expressing the desired proteins was centrifuged to a pellet at 12000g for 20 min at 4 °C, and the supernatant was removed and discarded. The bacterial cell pellet was resuspended in 15 mL of lysis buffer (50 mM NaH_2PO_4 , 30 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by sonication, on ice, using a programmed cycle of 10 s on, 10 s off, for 10 min total. The cell debris was pelleted by centrifugation 12000g for 20 min at 4 °C, and the resulting crude protein-containing cell extract was removed to a separate culture tube. To the crude extract was added 1.0 mL of Ni-NTA resin, and this was mixed at 4 °C for 1 h. The solution was then added to a gravity-flow column, and the flow through was collected. The resin was washed with 20 mL of wash buffer (50 mM NaH_2PO_4 , 30 mM NaCl, 20 mM imidazole, pH 8.0), and the wash fraction was collected. Purified protein was eluted from the column in 1.0 mL aliquots of elution buffer (50 mM NaH_2PO_4 , 30 mM NaCl, 250 mM imidazole, pH 8.0). All collected fractions were analyzed by SDS-PAGE electrophoresis, and fractions containing purified protein were combined and stored at 4 °C.

uGRPs Expression and Purification. Plasmid pQE70 with the gene encoding the full length GBP/tGRPs containing a unique Cys and pLacl were co-transformed into the *E. coli* tryptophan auxotroph ATCC no. 27873 for expression of uGRPs-FW. Plasmid pQE70 with the gene encoding the full length GBP/tGRPs containing a unique Cys were transformed into the *E. coli* leucine auxotroph HB101F' for

expression of uGRPs-FL. Protein expression was performed using the medium shift method. A single freshly transformed colony was used to inoculate 5 mL of M9 media supplemented with 0.4% glucose, 1 mM MgSO_4 , 0.1 mM CaCl_2 , 1 mM thiamine, 0.1 volume of a solution containing 0.01% (w/v) each of 19 amino acids (-Leu/or -Trp), 40 $\mu\text{g mL}^{-1}$ Leu or Trp, and 100 $\mu\text{g mL}^{-1}$ ampicillin. This culture was allowed to grow overnight at 37 °C, 250 rpm. Then 500 mL of media containing the same ingredients was inoculated with the overnight grown 5 mL culture. This culture was then grown until the OD_{600} was ~ 0.5 – 0.6 . The cells were then centrifuged at 10,000 rpm for 10 min at 25 °C. The supernatant was discarded, and the cells were resuspended in a 0.9% NaCl solution for washing, and this was repeated three times. The cells were then resuspended in 500 mL of M9 minimal media which was supplemented with 0.4% glucose, 1 mM MgSO_4 , 0.1 mM CaCl_2 , 1 mM thiamine, 0.01% (w/v) each of 19 amino acids (-Leu/or -Trp), 100 $\mu\text{g mL}^{-1}$ ampicillin, and grown at 37 °C for 30 min. One millimolar IPTG was then added with 0.1 mM 5,5,5-fluoroleucine or 5-fluorotryptophan to the culture and was grown overnight at 37 °C. The cells were harvested by centrifugation, and the protein was purified using Ni-NTA resin as described for the expression of tGRPs. The purified protein was dialyzed against 3 changes of 10 mM HEPES, 0.2 mM CaCl_2 , pH 7.2.

t/u-GRPs Modification with Fluorophore. Purified proteins were reacted with an excess of DTT to reduce possible disulfide bonds. Excess DTT was removed by dialysis in 3500 MWCO dialysis cassettes in dialysis buffer (10 mM HEPES, 0.2 mM CaCl_2 , pH 8.0). The dialyzed protein was reacted with a 10-fold molar excess of MDCC dissolved in DMSO, using manufacturer's instructions. The labeling reaction was carried out overnight at 4 °C in an amber glass vial, protected from light. Following the labeling reaction, the protein was extensively dialyzed with dialysis buffer (as above) to remove any excess MDCC. Labeled proteins were stored at 4 °C, protected from light.

Assay of Glucose with MDCC-Labeled u/tGRPs. For the glucose assay, MDCC-labeled protein was used at a final concentration of 1×10^{-7} M in assay buffer (10 mM HEPES, 0.2 mM CaCl_2 , pH = 8.0). Glucose standards were freshly prepared in assay buffer by serially dilution using a freshly prepared 0.1 M glucose solution. For the assay, 180 μL of the labeled protein solution was added to 20 μL of each standard, as well as a blank, in triplicate and mixed gently and thoroughly, and fluorescence was measured immediately in a 200 μL quartz microcuvette. MDCC was excited at a wavelength of 419 nm, and fluorescence emission spectra was collected at an emission wavelength of 466 nm. Instrument slit widths were set to 4 nm, step size was 0.5 nm, integration time was 0.1 s, and the number of averages was set to 1. Emission maxima for each data point were graphed versus glucose concentration and analyzed using GraphPad Prism 5.0 software.

Mass Spectrometry (MS) of uGRPs. SDS Page gel pieces of all proteins with unnatural amino acids incorporated were digested with trypsin, and LC-ESI-MS/MS was performed using a ThermoFinnigan LTQ. Resulting MS/MS spectra were searched against proteins in the Swiss-Prot database using the mascot search engine (Matrix Science). The difference in mass of full length GBP or tGRPs and uGRPs corresponding to the mass of fluorines confirmed the incorporation of the unnatural amino acids.

Measurement of Far- and Near-UV Circular Dichroism (CD) Spectra. Far-UV and near-UV CD spectra were collected for tGRPs in both the presence and absence of glucose. Briefly, protein concentration was determined according to the method by Greenfield et al.²⁷ Proteins were dialyzed in CD buffer (10 mM phosphate, 0.2 mM CaCl_2 , pH 7.5). Protein samples of tGRP1 and tGRP2 were prepared at a concentration of 0.2 mg mL^{-1} with and without the presence of 100 mM glucose in CD buffer for far-UV experiments and 1.0 mg mL^{-1} for near-UV experiments. Samples of the remaining proteins for analysis of far-UV CD were prepared at the following concentrations: 0.18 mg mL^{-1} for uGRP-FW, 0.29 mg mL^{-1} for uGRP1-FW, 0.20 mg mL^{-1} for uGRP2-FW, 0.17 mg mL^{-1} for uGRP-FL, 0.18 mg mL^{-1} for uGRP1-FL, and 0.29 mg mL^{-1} for uGRP2-FL. Blank solutions consisting of CD buffer were prepared with and

without 100 mM glucose. Spectra were collected using a Jasco J-810 spectropolarimeter.

For far-UV experiments, spectra were collected at RT from 200 to 260 nm, the data pitch was set to 0.5 nm, scanning mode was set to continuous, scan speed was 20 nm/s, response was set to 8 s, bandwidth was 1 nm, and accumulations was set to 3. A quartz CD cuvette with a path length of 0.1 cm was used. Near-UV experiments were carried out at RT in a quartz cuvette with a 1.0 cm path length. Scans were performed with settings mentioned above; however, the wavelength range was 250–350 nm. Data for corresponding blanks were subtracted from each sample, and the results were converted to molar ellipticity. Spectra were created by plotting molar ellipticity versus wavelength using GraphPad Prism 5.0 software.

Determination of Protein Thermal Stability. The melting temperature of each of the GRPs was determined by monitoring CD at a wavelength of 222 nm. Samples were prepared as described for far-UV experiments. Settings were the same as above. The temperature range was set from 10 to 90 °C at a slope of 0.5 °C/min. Data was plotted versus temperature using GraphPad Prism 5.0 software and normalized with respect to percent unfolding.

Preparation of Hydrogel Precursor Solution and Optical Fiber Surface Modification. A hydrogel precursor solution was made from the combination of 8 μL of (1.125 mg of acrylamide and 4.125 μg of *N,N*-ethylenebis(acrylamide) in 1 mL), 4 μL of 10% glycerol, 9 μL of 1.2×10^{-4} M GRP-MDCC-acrylic acid, and 0.5 μL 2,2-diethoxyacetophenone. The fiber (core diameter of 200 μm) was silanized by immersing in MPTS (3-(trimethoxysilyl)propyl methacrylate) for 1 h at RT and then air-dried; 0.3 μL of hydrogel precursor solution was deposited on the tip of a silanized fiber and polymerized under UV for 30 min. The prepared fibers were stored at 4 °C until further use.

Sensor Response. The response of the fiber-based GBP152/GRPs-FW/FL hydrogel sensor was determined by immersing the tip of the hydrogel-coated fiber in 2 mL of glucose (2–20 mM) solutions containing 10 mM HEPES and 0.2 mM CaCl_2 with pH 8.0. The hydrogel was washed with 2 mL of buffer for 2 min after each concentration of glucose. The fluorescence intensity of the hydrogel with MDCC-labeled proteins at 475 nm, excited by the optical fiber at 425 nm, was recorded by Ocean Optics Spectrometer. Similarly, the sensor response was also determined in human serum and in pig blood, both spiked with different glucose concentrations (2–20 mM).

■ ASSOCIATED CONTENT

📄 Supporting Information

This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: SDaunert@med.miami.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge Jack Goodman at the University of Kentucky Mass Spectrometry Facility (UKMSF) for performing LC-ESI-MS/MS. This work was supported in part by grants from the National Institutes of Health, the National Science Foundation, and the National Aeronautics and Space Administration. S.J. is grateful to the Research Challenge Trust Fund (RCTF) of the Commonwealth of Kentucky for financial support. S.D. is grateful for financial support by the Gill Eminent Professorship from the University of Kentucky and the Lucille P. Markey Chair from the Miller School of Medicine of the University of Miami.

■ REFERENCES

- (1) Yoo, E.-H., and Lee, S.-Y. (2010) Glucose biosensors: An overview of use in clinical practice. *Sensors* 10, 4558–4576.
- (2) Wang, J. (2007) Electrochemical glucose biosensors. *Chem. Rev.* 108, 814–825.
- (3) Khan, F., Saxl, T. E., and Pickup, J. C. (2010) Fluorescence intensity- and lifetime-based glucose sensing using an engineered high- K_d mutant of glucose/galactose-binding protein. *Anal. Biochem.* 399, 39–43.
- (4) Salins, L. L. E., Ware, R. A., Ensor, C. M., and Daunert, S. (2001) A novel reagentless sensing system for measuring glucose based on the galactose/glucose-binding protein. *Anal. Biochem.* 294, 19–26.
- (5) Khan, F., Gnudi, L., and Pickup, J. C. (2008) Fluorescence-based sensing of glucose using engineered glucose/galactose-binding protein: a comparison of fluorescence resonance energy transfer and environmentally sensitive dye labelling strategies. *Biochem. Biophys. Res. Commun.* 365, 102–106.
- (6) Teasley Hamorsky, K., Ensor, C. M., Wei, Y., and Daunert, S. (2008) A bioluminescent molecular switch for glucose. *Angew. Chem., Int. Ed.* 47, 3718–3721.
- (7) Amis, T. J., Sherman, D. B., Nycz, C. M., Andaluz, S. A., and Pitner, J. B. (2007) Engineering and rapid selection of a low-affinity glucose/galactose-binding protein for a glucose biosensor. *Protein Sci.* 16, 2350–2359.
- (8) Sakaguchi-Mikami, A., Taneoka, A., Yamoto, R., Ferri, S., and Sode, K. (2008) Engineering of ligand specificity of periplasmic binding protein for glucose sensing. *Biotechnol. Lett.* 30, 1453–1460.
- (9) Ha, J.-S., Song, J. J., Lee, Y.-M., Kim, S.-J., Sohn, J.-H., Shin, C.-S., and Lee, S.-G. (2007) Design and application of highly responsive fluorescence resonance energy transfer biosensors for detection of sugar in living *Saccharomyces cerevisiae* cells. *Appl. Environ. Microbiol.* 73, 7408–7414.
- (10) Jäckel, C., Kast, P., and Hilvert, D. (2008) Protein design by directed evolution. *Annu. Rev. Biophys.* 37, 153–173.
- (11) Lin, F.-P., Chuang, H.-H., Liu, Y.-H., Hsieh, C.-Y., Lin, P.-W., and Lin, H.-Y. (2009) Effects of C-terminal amino acids truncation on enzyme properties of *Aeromonas caviae* D1 Chitinase. *Arch. Microbiol.* 191, 265–273.
- (12) Demishtein, A., Karpol, A., Barak, Y., Lamed, R., and Bayer, E. A. (2010) Characterization of a dockerin-based affinity tag: application for purification of a broad variety of target proteins. *J. Mol. Recognit.* 23, 525–535.
- (13) Vyas, N., Vyas, M., and Quioco, F. (1988) Sugar and signal-transducer binding sites of the *Escherichia coli* galactose chemoreceptor protein. *Science* 242, 1290–1295.
- (14) Torrice, M. M., Bower, K. S., Lester, H. A., and Dougherty, D. A. (2009) Probing the role of the cation- π interaction in the binding sites of GPCRs using unnatural amino acids. *Proc. Natl. Acad. Sci. U.S.A.* 106, 11919–11924.
- (15) Cirino, P. C., Tang, Y., Takahashi, K., Tirrell, D. A., and Arnold, F. H. (2003) Global incorporation of norleucine in place of methionine in cytochrome P 450 BM-3 heme domain increases peroxxygenase activity. *Biotechnol. Bioeng.* 83, 729–734.
- (16) Cisnetti, F., Gateau, C., Lebrun, C., and Delangle, P. (2009) Lanthanide(III) complexes with two hexapeptides incorporating unnatural chelating amino acids: Secondary structure and stability. *Chem.–Eur. J.* 15, 7456–7469.
- (17) Tang, Y., and Tirrell, D. A. (2001) Biosynthesis of a highly stable coiled-coil protein containing hexafluoroleucine in an engineered bacterial host. *J. Am. Chem. Soc.* 123, 11089–11090.
- (18) Wong, C.-Y., and Eftink, M. R. (1997) Biosynthetic incorporation of tryptophan analogues into staphylococcal nuclease: Effect of 5-hydroxytryptophan and 7-azatryptophan on structure and stability. *Protein Sci.* 6, 689–697.
- (19) Bilgiçer, B., Fichera, A., and Kumar, K. (2001) A coiled coil with a fluorine core. *J. Am. Chem. Soc.* 123, 4393–4399.
- (20) Tang, Y., Ghirlanda, G., Petka, W. A., Nakajima, T., DeGrado, W. F., and Tirrell, D. A. (2001) Fluorinated coiled-coil proteins prepared in vivo display enhanced thermal and chemical stability. *Angew. Chem., Int. Ed.* 40, 1494–1496.
- (21) Son, S., Tanrikulu, I. C., and Tirrell, D. A. (2006) Stabilization of bzipp peptides through incorporation of fluorinated aliphatic residues. *ChemBioChem.* 7, 1251–1257.
- (22) Voloshchuk, N., Zhu, A. Y., Snyder, D., and Montclare, J. K. (2009) Positional effects of monofluorinated phenylalanines on histone acetyltransferase stability and activity. *Bioorg. Med. Chem. Lett.* 19, 5449–5451.
- (23) Hodges, J. A., and Raines, R. T. (2003) Stereoelectronic effects on collagen stability: The dichotomy of 4-fluoroproline diastereomers. *J. Am. Chem. Soc.* 125, 9262–9263.
- (24) Beatty, K. E. and Tirrell, D. A. (2009) Noncanonical Amino Acids in Protein Science and Engineering, in *Protein Engineering* (Köhler, C. and RajBhandary, U. L., Eds.) pp 127–153, Springer, Berlin, Heidelberg.
- (25) Vyas, N. K., Vyas, M. N., and Quioco, F. A. (1988) Sugar and signal-transducer binding sites of the *Escherichia coli* galactose chemoreceptor protein. *Science* 242, 1290–1295.
- (26) Siegrist, J., Kazarian, T., Ensor, C., Joel, S., Madou, M., Wang, P., and Daunert, S. (2010) Continuous glucose sensor using novel genetically engineered binding polypeptides towards in vivo applications. *Sens. Actuators, B* 149, 51–58.
- (27) Greenfield, N. J. (2007) Using circular dichroism spectra to estimate protein secondary structure. *Nat. Protoc.* 1, 2876–2890.