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Engineered PQQ-Glucose Dehydrogenase as a Universal Biosensor Platform

Zhong Guo,[†] Lindy Murphy,[‡] Viktor Stein,[†] Wayne A. Johnston,[†] Siro Alcala-Perez,[‡] and Kirill Alexandrov*[,†]

[†]Institute for Molecular Bioscience and Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, Brisbane, QLD 4072, Australia

[‡]Molecular Warehouse Ltd., 40 Occam Road, Guildford GU2 7YG, U.K.

Supporting Information

ABSTRACT: Biosensors with direct electron output hold promise for nearly seamless integration with portable electronic devices. However, so far, they have been based on naturally occurring enzymes that significantly limit the spectrum of detectable analytes. Here, we present a novel biosensor architecture based on analyte-driven intermolecular recombination and activity reconstitution of a re-engineered component of glucometers: PQQ-glucose dehydrogenase. We demonstrate that this sensor architecture can be rapidly adopted for the detection of immunosuppressant drugs, α-amylase protein, or protease activity of thrombin and Factor Xa. The biosensors could be stored in dried form without appreciable loss of activity. We further show that ligand-induced activity of the developed biosensors could be directly monitored by chronoamperometry, enabling construction of disposable sensory electrodes. We expect that this architecture could be expanded to the detection of other biochemical activities, post-translational modifications, nucleic acids, and inorganic molecules.

The ability to rapidly monitor the concentrations and activities of biomolecules in complex samples is a key requirement in medicine, life sciences, and biotechnology. Protein-based biosensors have been used extensively to develop analytical and diagnostic applications. The increasing demand for point-of-care (POC) applications creates additional pressure to develop rapid, inexpensive, durable, and sensitive sensors capable of detecting multiple analytes.[1] Among all available readout systems, electrochemical sensors feature prominently due to their simplicity and specificity.[2] Electrochemical blood glucose sensors account for nearly 90% of the US$15 billion global biosensor market.[3] Screen-printed disposable electrodes functionalized with glucose dehydrogenase (GDH) or glucose oxidase are now ubiquitous and can be manufactured for less than $0.1 in a continuous screen-printing process.[4] The electron current generated by the biosensor enables its connectivity with portable electronic devices such as smart phones via inexpensive electronic adaptors. Remarkably, this technological and commercial success has not been paralleled by other electrochemical biosensors despite the need for better and cheaper diagnostics and analytics in many industries. While GDH has been used extensively to create new biosensor applications, this was achieved either by using the enzyme as a reporter in ELISA-type assays or by developing glucose-generating signaling circuits that ultimately activate GDH and induce electron flow.[5,6] The high current generated by this class of enzymes also enabled their use in implantable biofuel cells.[7] Recently, we engineered a PQQ-GDH-calmodulin chimer that functions as a Ca^{2+} biosensor.[8] Although the developed biosensor could be used for quantification of Ca^{2+} in biological samples and performed well when manufactured into a sensory electrode, the developed sensory architecture was not generic. Domain insertion is an inherently empiric process that relies on large conformational change of the sensory domain.[9] Such domains are readily available for only a few analytes, and their engineering is far from trivial.[10]

Analysis of the properties of the previously reported Ca^{2+}-sensing calmodulin-GDH chimer convinced us that GDH was a suitable building block for construction of synthetic electrochemical receptors. Its excellent physical stability combined with very high catalytic rate (k_{cat} = 3860/s) makes it an ideal actuator for direct interfacing with electronic devices.[11] The observation that the loop connecting strands A and B of the β-sheet 3 could tolerate insertion of large domains[8] prompted us to test whether the enzyme could be split at this position and used to construct a two-component sensory system (Figure 1A). To this end, we expressed N- and C-terminal portions of the enzyme in *E. coli* in fusion with FRB and FKBP domains that form a complex in the presence of rapamycin. While the FRB-GDH1-153 could be produced and purified to homogeneity, the FKBP-GDH155-454 formed inclusion bodies and could not be obtained in soluble form. This is not entirely surprising given the fact that the split exposes the hydrophobic core of the protein. Due to compromised integrity and stability, split proteins generally perform much better in vivo than in vitro, presumably due to the presence of chaperon systems in the former.[12]

We found potential in the fact that the N-terminal fragment of GDH was physically stable and apparently folded autonomously. We conjectured that if the C-terminal fragment could be stabilized in solution, we might be able to proceed with our original plan. We constructed a variant of GDH with the TVMV cleavage site in the loop connecting strands A and B and carrying a FKBP domain on its C-terminus. We also mutated the

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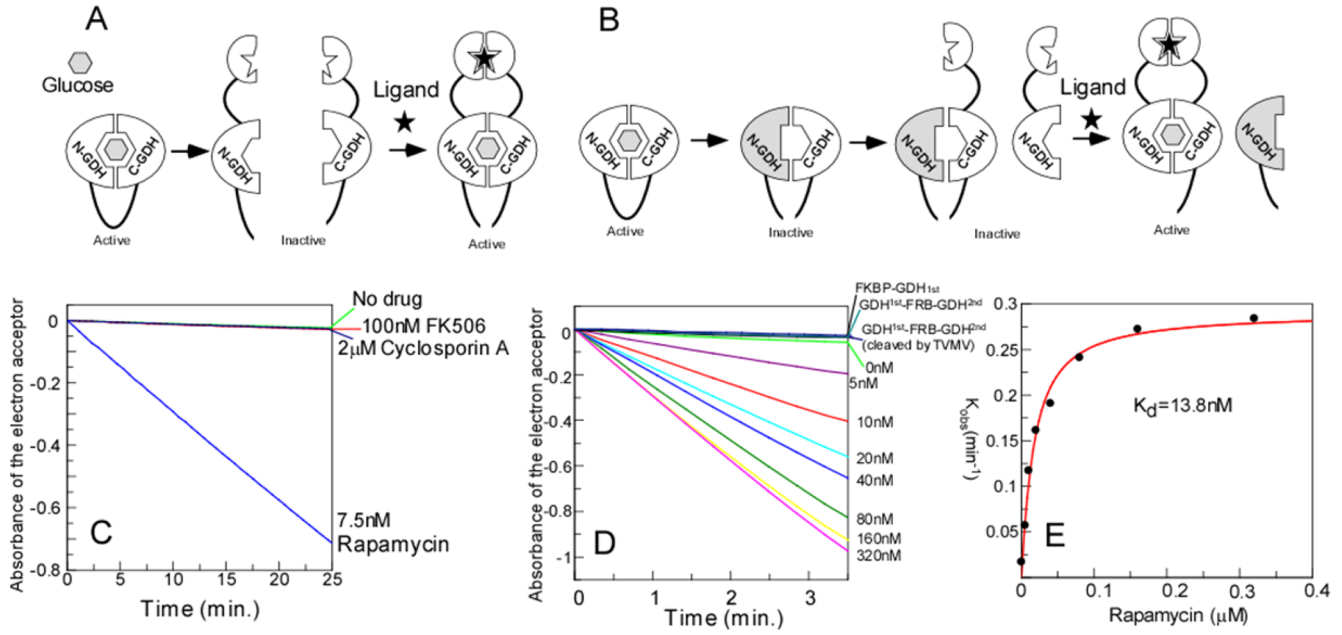

Figure 1. Converting PQQ-GDH into a two-component biosensor. (A) Strategy for splitting GDH into two inactive halves by disrupting the loop between β-strands A and B. The activity of the enzyme is then reconstituted by the ligand-mediated scaffolding (B), an extension of the strategy shown in A where the active site residues harbored by the N-terminal fragment are mutated to alanine and the loop connecting β-strands A and B are proteolyzed upon enzyme purification. (C) Change in the activity of the split GDH enzyme bearing rapamycin binding domains FRB and FKBP in the presence of rapamycin or other macrocyclic immunosuppressants such as FK506 or cyclosporin A. The activity of the enzyme was monitored by change of 600 nm absorption of 60 μM electron-accepting dye dichlorophenolindophenol in the presence of 0.6 mM electron mediator phenazine methosulfate, 20 mM of glucose, 15 nM of FRB-GDH1-153, 10 nM of inactive full-length GDH-FKBP12, and 50 μM $CaCl_2$. (D) Same as in C but using increasing concentrations of rapamycin. (E) Fit of the observed reaction rates obtained in D to a quadratic equation (see Supporting Information for details).

catalytically important residues Gln76, Asp143, and His144 in the active site to Ala, rendering the fusion protein catalytically dead (Figure 1B). The resulting recombinant protein was soluble and displayed no detectable catalytic activity. When the fusion protein was mixed with the purified FRB-GDH1-153 in the presence and in the absence of rapamycin, little activity was recovered. We then digested the protease cleavage site in the loop connecting N and C fragments of GDH and repeated the reconstitution experiment. As in the previous case, little GDH activity was recovered. However, when the system was exposed to rapamycin, we observed rapid and dose-dependent recovery of GDH activity, indicating that the inactivated N-terminal fragment of GDH was displaced by the FRB-fused GDH1-153 fragment (Figure 1C). We titrated the reaction with increased concentrations of rapamycin and fitted the observed rates to a K_d value of 11 nM that is very close to the previously published value (Figure 1D,E).[13] In the fully activated state, the resulting biosensor displayed approximately 20% of the catalytic rate of the wt enzyme (Figure S1). However, due to the very high turnover rate of the parent enzyme, it was possible to obtain satisfactory response to low nanomolar concentrations of the analyte on the minute scale (Figures 1B and S1). Importantly, the reaction was specific as the excess of the related immunosuppressant compounds such as FK506 or cyclosporine did not activate the biosensor to an appreciable degree (Figure 1C).

We next tested whether the developed sensor architecture was sufficiently generic and could be expanded onto the other small molecules and biomarkers. As rapamycin belongs to the class of clinically important macrocyclic immunosuppressants, together with cyclosporine and FK506 that do not cross-react with the developed biosensor, we wondered if similar biosensors could be developed for these drugs, as well. We were additionally motivated by the fact that the immunosuppressants have a very narrow therapeutic window, and no POC test for these compounds currently exists.[14] We analyzed the available cocrystal structures of cyclosporine with calcinurin A and B and peptidylprolyl *cis–trans* isomerase (cyclophilin) (PDB: 1MF8) and FK506 in complex with calcinurin A and B and FKBP (PDB: 1TCO). The topology of the complex allowed us to design fusion proteins of GDH fragments that were expected to come into the molecular proximity when the complex assembly is induced by a ligand. The fusion proteins were produced in recombinant and purified form (Table S1), and their mixtures responded to the cognate drug in a dose-dependent manner (Figures 2A and S2). These results demonstrate that the developed biosensor architecture could be rapidly adopted to detection of xenobiotics with known targets.

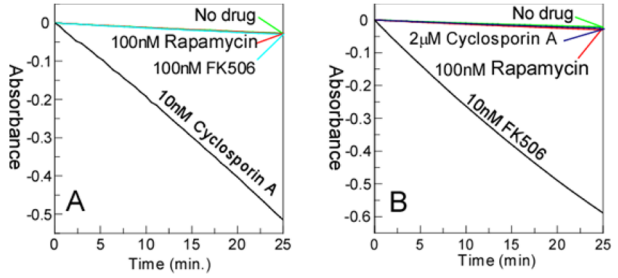

Figure 2. Developing biosensors for cyclosporin A and FK506 (A) activation of the cyclosporin A biosensor (15 nM N-terminal GDH-Calβ/Calα complex and 10 nM inactive CYPA-GDH fusion) by cyclosporin but not FK506 or rapamycin. (B) Using FK506 biosensor (15 nM N-terminal GDH-Calβ/Calα complex and 10 nM inactive CYPA-GDH fusion).

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In the next step, we wanted to test whether the developed biosensor architecture could be applied to the detection of protein biomarkers. As the first example, we chose human stress biomarker salivary α-amylase. We analyzed the available crystal structures of α-amylase and identified three crystal structures of porcine. Based on structural analysis of α-amylase bound to VHH domains (PDB: 1KXQ, 1KXT, 1KXV, and 1BVN), we constructed fusion proteins with GDH1-153-VHH1KXV and GDH inactive mutant with insertion of VHH1BVN and produced them in recombinant form (Figure 3A). Addition of

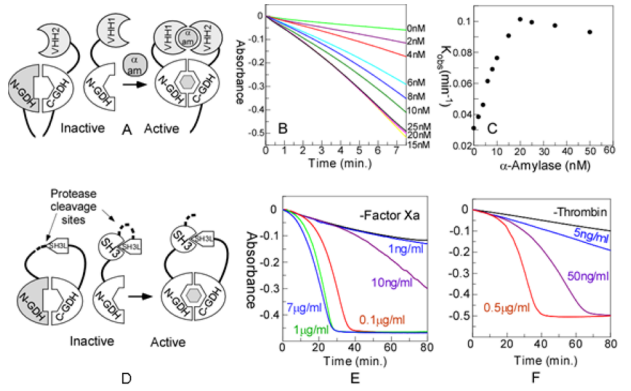

Figure 3. Adapting the developed biosensory architecture for detection of proteins and enzymatic activities. (A) Schematic representation of a two-component α-amylase (α-am) biosensor. (B) Change in enzymatic activity of the biosensor at different concentrations of α-amylase; 30 nM of GDH1-153-VHH_{1KXV} and 20 nM of inactive of full-length GDH with VHH_{1BVN} insertion were used in the assay. (C) Plot of the observed rates against the concentration of amylase. (D) Schematic representation of a protease biosensor based on GDH, where reconstitution is driven by the interaction of the SH3 domain with its ligand peptide (SH3L). (E) Increase in GDH activity of the Factor Xa biosensor in the presence of increasing concentrations of Xa protease; 15 nM of fusion protein GDH1-153-SH3-SH3 binding peptide and 10 nM of inactive of full-length GDH with insertion of SH3 binding peptide were used in the assay. (F) Same as in E but using the biosensor bearing a thrombin cleavage site.

human salivary α-amylase to the mixture of both recombinant proteins resulted in a dose-dependent increase in GDH activity that decreased at higher concentration, reflecting formation of α-amylase complexes with only one fusion protein bound to it (Figure 3B,C). These results demonstrate that the developed sensory architecture could be used for the detection of protein biomarkers.

Finally, we wanted to establish whether the developed sensor architecture could be used to measure enzymatic activities such as proteolysis rather than molecular entities. To connect the protease activity to reconstitution of GDH from fragments, we created an autoinhibited version of the SH3 domain in which an SH3 domain binding peptide was linked to SH3 domain via a protease-digestible linker (Figure 3D). In this configuration, the SH3 domain is protected from the binding of an external SH3 domain binding peptide as long as the linker connecting the domain and its ligand is intact. We fused the autoinhibited SH3 module to GDH1-153 C-terminally, while SH3 peptide was inserted into inactive full-length GDH with protease cleavage site at its N-terminal. In this design, we placed a Factor Xa cleavage site between the SH3 domain and its ligand and between the inactive N-terminal of GDH1-153 and SH3 binding peptide. When the solution of the constructs produced in the recombinant form were mixed together, only low level GDH activity could be detected, suggesting that SH3 peptide was not able to drive enzyme reconstitution. However, addition of Factor Xa resulted in time-dependent increase of GDH activity, indicating that proteolytic removal of the autoinhibitory peptide enables binding of the SH3 peptide in *trans* and reconstitution of the active complex (Figure 3E).

To demonstrate the universal nature of the protease biosensor, we replaced the Factor Xa cleavage site with that of thrombin. We repeated the same experiment using a thrombin cleavage site and thrombin protease and observed a robust activation of the biosensor in the presence of the protease (Figure 3F). When comparing the response rate of the protease biosensor to that of immunosuppressant and amylase, we noticed that the former was activated significantly slower. We suspected that this was due to the high K_m and low k_{cat} of proteases, resulting in comparatively slow rates of linker digestion. Incorporation of the three copies of the Factor Xa substrate sequence increased both the response rate and the sensitivity of this biosensor presumably by reducing the complete dissociation of nonproductive protease/peptide complexes (Figure S3).

The overwhelming success of the GDH-based glucose monitors is at least in part due to the high stability of the enzyme that allows dehydration of the biosensor on the electrodes and their long-term storage at ambient temperatures. To test whether the engineered versions of GDH could be desiccated and rehydrated in functional form, we incubated dried rapamycin biosensor for up to 2 weeks at room temperature (Figure S4) with no appreciable loss of activity upon reconstitution. In an alternative experiment, the dried biosensor was incubated for 2 h at different temperatures. Data show that no activity was lost up to 40 °C, and only a small reduction in activity was observed up to 50 °C (Figure S4). Next, we tested the performance of the α-amylase biosensors in chronoamperometric measurements using a commercial potentiostat. As seen in Figure S5A,B, the biosensor generated progressively higher current when exposed to the increasing concentrations of α-amylase.

To date, only electrochemical biosensors such as the glucose monitors meet the performance and cost benchmarks for their ubiquitous deployment. The success of glucose monitors relates to the prevalent and the debilitating nature of the diabetic condition, the abundance of the analyte that simultaneously serves as a source of energy, and the remarkable stability of the biosensor. Here, we build on the highly advanced glucometer technology by re-engineering to its principle catalyst PQQ-GDH into a generic biosensor architecture. By exploiting the remarkable biophysical stability of the enzyme, we convert it into a two-component signaling system where individual components are neither active nor capable of reassembly (Figure 1B). This is achieved by creating a split enzyme with inactive fragment that serves as an inhibitor of spontaneous activation and as a chaperon factor ensuring the correct folding and stability of the active half of the enzyme. A somewhat similar strategy was recently devised to stabilize a split fibronectin receptor system where ligand binding to its cognate fibronectin receptor drove the functional association of two split fragments, which was subsequently actuated through a fluorescent signal.[15] Yet, no such strategy has been devised for split enzyme sensors that often suffer from inferior biophysical properties, limiting their utility for in vitro applications, as recently demonstrated for firefly luciferase.[16] Activation of the developed system is achieved through the analyte-mediated scaffolding of the fragments that

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result in the concentration-driven replacement of the inactive N-terminal fragment with its active form. The kinetics of activation is surprisingly rapid, indicating high off rate of the engineered complex. The sensors demonstrated excellent response rate, sensitivity, and selectivity, confirming that the system is modular and can be adopted practically for any analyte for which a binding domain can be found. In principle, the system can be expanded to semisynthetic systems and used to detect association events mediated by small molecules, nucleic acids, and other types of biological molecules and post-translational modifications. This has potentially important implications as the ubiquitous presence of the Internet-enabled personal electronic devices has long supported the idea of personal mobile analytic and diagnostic applications that already stimulated the efforts to create biosensors based on redox enzymes.[17,18] The high catalytic rate of GDH-based biosensors and the resulting high electron current simplify the detection of the molecular recognition events, potentially enabling electrode miniaturization and multiplexing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06342.

Sequences and expression conditions for biosensors used in this work, assay conditions, data processing methods, and Figures S1−S5 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*k.alexandrov@uq.edu.au

Notes

The authors declare the following competing financial interest(s): Kirill Alexandrov and Siro Perez hold equity in Molecular Warehouse Ltd.

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