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United States Patent Application**20190264253****Kind Code****A1****GUO; Zhong ; et al.****August 29, 2019****ULTRASENSITIVE ELECTROCHEMICAL BIOSENSORS****Abstract**

The invention relates to biosensors. More particularly, this invention relates to an electrochemical biosensor and to electrochemically active enzymes or variants thereof that are suitable for detection of one or more target molecules in a sample.

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Claims

1. An oxidoreductase enzyme comprising a heterologous amino acid sequence which is responsive to a peptide, wherein binding of the peptide to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme.
2. The oxidoreductase enzyme of claim 1, which displays a reduction in catalytic activity in the absence of binding of said peptide to the heterologous amino acid sequence.
3. The oxidoreductase enzyme of claim 1 or 2, wherein the heterologous amino acid sequence is a calmodulin protein, or a functional fragment thereof.
4. The oxidoreductase enzyme of claim 3, which is responsive to a calmodulin-binding peptide, wherein binding of the peptide activates catalytic activity of the enzyme.
5. The oxidoreductase enzyme of claim 1, which displays a reduction in catalytic activity in the presence of binding of said peptide to the heterologous amino acid sequence.
6. The oxidoreductase enzyme of claim 5, wherein the heterologous amino acid sequence is an affinity clamp which binds said peptide.
7. The oxidoreductase enzyme of claim 5 or 6, which is responsive to a target molecule, wherein binding of the target molecule displaces said peptide to activate catalytic activity of the enzyme.
8. The oxidoreductase enzyme of any one of the preceding claims, comprising a binding moiety capable of interacting with a respective binding moiety on said peptide, wherein interaction between the binding moieties regulates catalytic activity of the enzyme.
9. The oxidoreductase enzyme of any one of claims 1 to 4, comprising a binding moiety capable of interacting with a respective binding moiety on said peptide, wherein catalytic activity of the enzyme is cooperatively enhanced by binding of the peptide and interaction between the binding moieties.
10. The oxidoreductase enzyme of claim 8 or 9, wherein said peptide is engineered to bind the heterologous amino acid sequence with an affinity insufficient to enhance catalytic activity in the absence of said interaction between binding moieties.
11. The oxidoreductase enzyme of any one of claims 8 to 10, wherein interaction of the binding moieties is dependent on presence of a target molecule, such that catalytic activity of the enzyme is enhanced in the presence of the target molecule.
12. The oxidoreductase enzyme of any one of the preceding claims, comprising one or more protease cleavage sites, wherein cleavage of a said site by a protease acts to regulate catalytic activity of the enzyme.
13. The oxidoreductase enzyme of any one of the preceding claims, wherein said peptide is covalently attached to, or forms part of a contiguous amino acid sequence of, said enzyme.
14. The oxidoreductase enzyme of claim 13, wherein said peptide is incapable of binding the heterologous amino acid sequence in the absence of a further molecule.
15. The oxidoreductase enzyme of claim 14, which comprises a moiety acting to prevent binding of said peptide to the heterologous amino acid sequence, wherein said moiety is displaced in the presence of the further molecule.
16. The oxidoreductase enzyme of claim 15, wherein said moiety is a binding moiety capable of interacting with a respective binding moiety on said further molecule, wherein interaction between the binding moieties releases said peptide to bind to the heterologous amino acid sequence.
17. The oxidoreductase enzyme of claim 15 or 16, wherein said moiety comprises one or more protease

41. A nucleic acid encoding the oxidoreductase enzyme of any one of claims 1 to 23, a polypeptide comprising a first or second fragment sequence as defined in any one of claims 24-29, the biosensor comprising an enzyme of claim 30, or the biosensor of claim 31.

[0002] Detection of target molecules or analytes in biological samples is central to diagnostic monitoring of health and disease. Key requirements of analyte detection are specificity and sensitivity, particularly when the target molecule or analyte is in a limiting amount or concentration in a biological sample. Previous approaches include use of monoclonal antibodies which specifically bind the analyte. This type of diagnostic approach has become well known and widely used in the enzyme-linked immunosorbent sandwich assay (ELISA) format which is the gold standard for detecting specific analytes in complex biological samples.

[0003] Over the last three decades, biosensors have also become a practical alternative to complex and expensive analytical instruments used in healthcare, agriculture and environmental monitoring.^{sup.1} Among several currently used detection technologies such as optical, acoustic and piezoelectric, electrochemical sensors feature prominently due to their simplicity, specificity and high performance.^{sup.2} Electrochemical blood glucose sensors are the most commercially successful biosensors accounting for nearly 90% of the US\$15 billion global biosensor market (Transparency Market Research report, titled, 'Biosensors Market--Global Industry Analysis, Size, Share, Growth, Trends and Forecast, 2014-2020'). The success of these sensors is due to their high selectivity and sensitivity combined with simplicity of design and ease of manufacturing. The sensors are based on amperometric monitoring of glucose oxidation by recombinant glucose oxidase or glucose dehydrogenase (GDH).^{sup.3} The simplicity and robustness of the design enables manufacturing of disposable glucose-sensing electrodes for less than \$0.1 in a continuous screen printing process.^{sup.4} The electrochemical output of the biosensor enables its connectivity with portable electronic devices such as smart phones via unsophisticated and 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. This can be at least in part explained by the unique features of glucose sensing where the analyte is present at high (4-10 mM) concentration which also provides the source of energy for a selective, physically stable and highly processive electrochemical receptor.

[0004] There is a need for biosensors which are able to detect other analytes than glucose, in particular for biosensors which may be configured to detect a range of different analytes, and also for biosensors which provide increased sensitivity of detection.

SUMMARY

[0005] The present invention addresses a need to develop quantitative, relatively inexpensive and easily produced molecular biosensors that readily detect the presence or the activity of target molecules (e.g analytes) on short time scales that are compatible with treatment regimes. Such biosensors can either be applied singly or in multiplex to validate and/or diagnose molecular phenotypes with high specificity and great statistical confidence irrespective of the genetic background and natural variations in unrelated physiological processes. Such biosensors may be used in other testing procedures such as where the target molecule or analyte is an illicit drug or performance-enhancing substance.

[0006] The biosensors of the present invention are further particularly suited to incorporation into electrical devices such as point-of-care devices for analysis and transmission of diagnostic results. The biosensors of the invention typically have specificity for a target molecule and produce an electrical response to detection of the target molecule. Preferred biosensors of the present invention provide high sensitivity to a target molecule through allosteric peptide-regulated reversible changes to catalytic activity which are further linked to binding of the target molecule to a binding moiety. The peptide-regulated change may be couplable to a range of different binding interactions thus allowing for detection of different target molecules based on the same common peptide-regulated architecture. The biosensors of the invention may thus be suitable for engineering for use in detection of more than one target molecule when configured with appropriate binding moieties. The biosensors of the invention typically comprise an oxidoreductase enzyme or a variant thereof.

[0007] The present invention provides an oxidoreductase enzyme comprising a heterologous amino acid sequence which is responsive to a peptide, wherein binding of the peptide to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme. The binding of the peptide may cause a reduction in the catalytic activity of the enzyme, or may enhance the catalytic activity of the enzyme.

[0008] The invention further provides an oxidoreductase enzyme comprising a heterologous amino acid sequence inserted at a location comprising one or more residues which influence substrate binding of said

[0009] The invention additionally provides a polypeptide comprising a first fragment sequence of an oxidoreductase enzyme, which is capable of non-covalently interacting with a polypeptide comprising a second fragment sequence of said enzyme to reconstitute a stable oxidoreductase enzyme, wherein the first and second fragment sequences represent sequences obtainable by cleavage of the enzyme at a location comprising one or more residues which influence substrate binding by said enzyme.

[0011] The invention also provides a biosensor comprising an oxidoreductase enzyme of the invention or the polypeptides comprising first and second fragment sequences of the invention.

[0013] The invention additionally provides a method of detecting a target molecule, comprising contacting the oxidoreductase enzyme of the invention, a biosensor of the invention or the polypeptides comprising first and second fragment sequences of the invention with a sample under conditions suitable for detection of the presence or absence of the target molecule in the sample.

[0015] The invention also provides a detection device that comprises a cell or chamber that comprises the oxidoreductase enzyme of the invention, a biosensor of the invention or the polypeptides comprising first and second fragment sequences of the invention.

BRIEF DESCRIPTION OF THE FIGURES

[0018] FIG. 2. Construction of CaM-BP (Calmodulin-binding peptide) inducible GDH-CaM chimera and its use for construction of a two component rapamycin biosensor. (A) schematic representation of a GDH-CaM

[0019] FIG. 3. Testing the generic nature of the two component biosensor architecture based on a CaM-GDH chimera. (A) Schematic representation of the FK506 (tacrolimus) biosensor. (B) Time resolved changes in GDH activity of 10 nM solution of GDH-CaM-FKBP12 fusion with 30 nM solution of CaM-BP calcinurin B fusion in complex with calcinurin A in the absence or presence of indicated concentrations of FK506. (C) Schematic representation of the α -amylase two component biosensor. (D) As in B but using 20 nM GDH-CaM-VHH fusion and 50 nM VHH-CaM-BP

[0021] FIG. 5. Development and applications of an OFF switch biosensor based on GDH. (A) Schematic representation of an affinity clamp operated GDH biosensor, L-denotes the ligand peptide, (B) Enzymatic activity of 10 nM solution of the affinity clamp-GDH chimera in the presence of 1 μ M of a strong or 2 μ M of a weak peptide ligand. (C) as in B but using the biosensor with the optimized linkers between the affinity clamp and the GDH, titrated with the increasing concentrations of the strong ligand peptide. (D) A dissociative biosensor architecture based on the developed affinity clamp-GDH biosensor. The star denotes the ligand either genetically fused or conjugated to the regulatory domain. (E) Exemplification of the design shown in D with a biosensor for IL18: the ligand is IL18 and the binder is IL18 binding protein. (F) Enzymatic activity of 10 nM solution of the affinity clamp-GDH IL18 binding protein chimera (SEQ ID NO: 37) mixed with 50 nM solution of the fusion of IL18 with the affinity-clamp ligand (SEQ ID NO: 38), at varying concentrations of IL18 (top to bottom traces from 0 to 2.5 μ M IL18). (G) As in D but using a binder that competitively associates with the receptor domain and is dislodged by the binding of the ligand.

[0023] FIG. 7. Comparison of the activation rates of the split and GDH-CaM-based versions of rapamycin biosensors. Split version: A solution of 10 nM of a fusion of mutational inactivated N-terminal GDH fragment fused to TVMV cleavage site-FKBP- and wild type C-terminal portion of GDH, as described in Examples 1 and 5 (SEQ ID NO: 44). The protein was pre-cleaved with TVMV prior to the assay. The former solution was mixed with 15 nM solution of N-terminal GDH-FRB, SEQ ID NO: 43. The GDH-CaM rapamycin biosensor was: 10 nM GDH-CalM-FKBP (SEQ ID NO: 11), 2.5 .mu.M FRB-CalM BP (SEQ ID NO:12).

[0052] SEQ ID NO: 29 is the amino acid sequence of a GDH-ePDZ-CaM-thrombin cleavage site-CaM-BP-PDZ binding peptide fusion protein.

[0071] An oxidoreductase "enzyme" is a protein capable of displaying catalytic activity towards a substrate molecule to thereby produce one or more electrons. The enzyme may be any enzyme capable of reacting with a substrate molecule to thereby produce one or more electrons. Preferably, the enzyme is an oxidoreductase such as a GDH, glucose oxidase, LDH or DHFR. In some embodiments, the enzyme is an oxidoreductase and the activity is oxidoreductase activity. Preferably the enzyme is glucose dehydrogenase (GDH) and the substrate molecule is glucose. The catalytic activity may thus be glucose dehydrogenase activity which may be measured in accordance with Example 1. The glucose dehydrogenase may be a PQQ-GDH or an FAD-GDH. Preferably, the GDH is a PQQ-GDH. A PQQ-GDH preferably comprises the sequence of SEQ ID NO: 1 or a variant thereof. A PQQ-GDH may be encoded by a nucleic acid sequence encoding SEQ ID NO: 1 or 2.

[0073] The oxidoreductase enzyme has a reduced or enhanced state of catalytic activity when the peptide is bound to the heterologous amino acid sequence. In some embodiments the oxidoreductase enzyme has a reduced or enhanced state of catalytic activity when the peptide is bound to the heterologous amino acid sequence, and a target molecule is also bound to a binding moiety. The reduction or enhancement of catalytic activity may be of any magnitude. The reduction or enhancement of catalytic activity is typically of a magnitude sufficient to allow for correlation with the presence of the peptide or the presence of both the peptide and target molecule. The skilled person is able to determine whether binding of the peptide regulates catalytic activity of the enzyme by comparing the activity of the enzyme with and without the peptide.

[0075] The oxidoreductase enzymes and biosensors described herein produce electrons by reacting with substrate molecules in response to binding, interacting with or otherwise detecting one or more target molecules. In this context "react", "reaction" or "reacting" with a substrate molecule means enzymatically transforming the substrate molecule into one or more product molecules with a net or overall production of one or a plurality of electrons per substrate molecule. Accordingly, the biosensor acts as an electron donor, whereby the electrons produced by the reaction may flow either directly or via an electron shuttle such as, but not limited to, phenazine methosulfate or potassium ferrocyanide, to thereby act as an anode. The resulting change in potential between anode and cathode may be detected by an electronic detector.

[0077] In some embodiments the oxidoreductase enzymes and biosensors described herein may be integrated into semiconductor electronic devices. Typically, the biosensor or enzyme acts as an electron donor and electrons produced by the reaction may flow either directly or via an electron shuttle such as, but not limited to, phenazine methosulfate or potassium ferrocyanide, to the semiconductor. The enzyme or biosensor may be integrated into an electrolyte-insulator-semiconductor (EIS) chip. For example, the enzyme or biosensor may be attached to a Ta.sub.2O.sub.5 surface. Ta.sub.2O.sub.5 is a pH-sensitive material and allows pH changes resulting from reaction of the biosensor or enzyme with a substrate to produce an acidic or basic product to be detected.

[0079] The heterologous amino acid sequence is typically provided as an insert within the amino acid sequence of the oxidoreductase enzyme. However fusions of the heterologous amino acid sequence at the N- or C-terminus are also possible.

[0080] Where provided as an insert, the heterologous amino acid sequence is therefore typically and contiguous with, respective portions, sub-sequences or fragments of said enzyme. The insertion is made at a position in the amino acid sequence of the enzyme which tolerates said insertion without steric clashes preventing stable folding of the enzyme. Linker sequences may be added between the insert and the sequence of the enzyme to assist toleration of the insertion. The insertion may be located at a loop or turn region in the structure of the enzyme which functionally tolerates the heterologous, sensor amino acid sequence. The insertion may be located in a region of the enzyme (such as a loop or turn region) which comprises one or more amino acid residues which influence substrate binding and/or catalytic activity of the enzyme. The insertion may thus displace one or more residues which influence substrate binding and/or catalytic activity of the enzyme, such that catalytic activity of the enzyme is regulated by the heterologous amino acid sequence. The insertion may displace one more residues which influence substrate binding and one or more residues which influence catalytic activity. The insertion may prevent or reduce substrate binding to the enzyme and/or may switch the enzyme to a state of reduced catalytic activity or a catalytically inactive state. As discussed above, the binding of the peptide to the heterologous amino acid sequence or of the peptide to the heterologous amino acid sequence and the target molecule to its binding moiety reversibly regulates catalytic activity of the enzyme. The binding of the peptide or of the peptide and the target molecule may thus reverse the displacement of one or more residues which influence substrate binding and/or catalytic activity of the enzyme by the heterologous amino acid sequence, or cause said displacement.

[0081] The catalytic activity of the enzyme may accordingly be regulated by the conformational status of the heterologous amino acid sequence, as affected by binding of the peptide or binding of the peptide and binding of the target molecule to its binding moiety. The insertion thus typically allows for the heterologous amino acid sequence to reversibly regulate catalytic activity through inducing a conformational change in the enzyme, typically at the substrate binding region and/or active site of the enzyme. The heterologous amino acid sequence typically undergoes a conformational change in the presence of the peptide or in the presence of the peptide and the target molecule which acts to regulate catalytic activity of the enzyme. The heterologous amino acid sequence may thus allosterically regulate the catalytic activity of the enzyme in the presence of the peptide or in the presence of the peptide and the target molecule.

[0082] In a preferred embodiment, the heterologous amino acid sequence is inserted in a GDH enzyme at a loop or turn region corresponding to the loop connecting beta-sheets 4 and 5 of PQQ-GDH of SEQ ID NO:1, or in a region corresponding to residues 326 to 335 of said enzyme. Preferably said insertion is made in a region corresponding to residues 328 to 332. Most preferably, the insertion is made at position 330 of SEQ ID NO:1 or at a corresponding position in another enzyme. The above insertion may delete the amino acid at

[0083] The heterologous amino acid sequence may be any binding moiety for any peptide. Exemplary heterologous amino acid sequences and peptides are described below. The heterologous amino acid sequence is preferably an amino acid sequence of a calcium-binding protein, or a functional fragment thereof. The calcium binding protein may be a calmodulin or a functional calcium-binding fragment thereof. The calcium-binding protein may be calmodulin of SEQ ID NO 3 or a variant thereof. Where the heterologous amino acid sequence is calmodulin of SEQ ID NO 3 or a variant thereof, the peptide binding thereto is typically a calmodulin-binding peptide. The calmodulin-binding peptide may comprise SEQ ID NO: 4 or a variant thereof. Variants thereof suitably retain calmodulin-binding activity, and may include any of SEQ ID NOs 5-8. Preferably, the calmodulin-binding peptide comprises or consists of SEQ ID NO 8, which has reduced calmodulin-binding activity, or a variant thereof. The invention also provides the above calmodulin-binding peptides and variants as peptides per se.

[0085] Where the heterologous amino acid sequence is a calcium-binding protein or functional fragment thereof, regulation of catalytic activity of the enzyme typically requires the presence of calcium ions in addition to the peptide, or in addition to the peptide and the target molecule. Such an oxidoreductase enzyme typically does not display regulation of catalytic activity by the heterologous amino acid sequence in the presence of calcium alone, absent the peptide or the peptide and the target molecule. The invention preferably provides an oxidoreductase enzyme based on PQQ-GDH and calmodulin as described below comprising SEQ ID NO: 9 or 10 or a variant thereof. An oxidoreductase enzyme comprising SEQ ID NO: 10 is particularly preferred.

[0087] The peptide may be any peptide binding moiety described herein which is able to bind to the heterologous amino acid sequence, including suitable peptides listed as "binding moieties" below. Preferred peptides include calmodulin-binding peptides and peptides binding affinity clamps as described herein and in the Examples. Other possible peptides include SH3:SH3 domain binding peptide, antibody: antibody binding

peptide, two leucine zipper peptides. The peptide may comprise a further binding moiety for a binding partner (respective binding moiety) other than the heterologous amino acid sequence, as discussed below. The peptide binding to the heterologous amino acid sequence may be provided as a separate molecule to the enzyme (and thus as a further component of a biosensor), or alternatively may form part of the contiguous amino acid sequence of said enzyme. In the latter embodiment, the peptide is located at a position from which it is able to interact with the heterologous amino acid sequence under conditions promoting such interaction. The peptide may be comprised as an insert within the amino acid sequence of the enzyme or as a C-terminal or N-terminal fusion thereto.

[0088] The oxidoreductase enzyme may further comprise respective binding moieties whose binding prevents interaction between the peptide and the heterologous amino acid sequence. Any pair of respective binding moieties may be used. This allows for autoinhibition of enzyme activity. Disruption of the interaction between the respective binding moieties can then provide for regulation of catalytic activity by the peptide. A ligand competing for binding with one of the respective binding moieties may be provided such that their interaction is disrupted. Multiple binding moieties may be provided such that disruption of more than one interaction is required for regulation of catalytic activity by the peptide, also reducing spontaneous activation. A protease cleavage site may be provided, suitably adjacent to one of the binding moieties, such that provision of a cognate protease allows for cleavage of the site to release the enzyme from autoinhibition by the interaction between the binding moieties. The peptide is thereby released to interact with the heterologous amino acid sequence.

[0089] Where the peptide is a calmodulin-binding peptide and the heterologous amino acid sequence is a calmodulin protein located in a GDH enzyme at a loop or turn region corresponding to the loop connecting beta-sheets 4 and 5 of PQQ-GDH of SEQ ID NO:1 (or a related location discussed above), the peptide may be provided C-terminally in the enzyme, preferably C-terminal to the native C-terminal GDH enzyme sequence. The invention provides in this embodiment a GDH enzyme comprising in order the sequences of SEQ ID NO: 30 (residues 1-329) or a variant thereof, the sequence of SEQ ID NO: 3 or a variant thereof, the sequence of SEQ ID NO: 31 (residues 331-454) or a variant thereof, and the sequence of SEQ ID NO 4 or a variant thereof, preferably SEQ ID NO: 8 or a variant thereof. The above sequences may be separated by linker sequences allowing for toleration of the inserted heterologous amino acid sequence as described above. The above series of sequences may be flanked N- and C-terminally by respective binding moieties preventing interaction between the peptide and the calmodulin protein. The N-terminal binding moiety may be the ePDZ domain of SEQ ID NO 26 and the C-terminal binding moiety the PDZ domain binding peptide of SEQ ID NO 27 or 28 or a variant of either thereof. In this embodiment, the invention further provides the oxidoreductase enzyme described below comprising SEQ ID NO: 29 or a variant thereof.

[0090] Target Molecule and Binding Moieties

[0091] Whilst the catalytic activity of the oxidoreductase enzyme of the invention may be solely regulated by binding of the peptide to the heterologous amino acid sequence, preferably it is further regulated by interaction of one or more binding moieties, typically further dependent on presence of a target molecule. In this manner, presence of a target molecule other than the peptide may be detected, with enhancement or reduction of catalytic activity then being indicative of the presence of the target molecule. Binding of the target molecule may cooperatively enhance regulation of the catalytic activity of the enzyme as described above.

[0092] The oxidoreductase enzyme may comprise a binding moiety and the peptide a respective binding moiety therefor such that interaction between the binding moieties regulates binding of the peptide to the heterologous amino acid sequence. The interaction between the binding moieties may induce a conformational change in the enzyme. The interaction between the binding moieties may further depend on the presence of a target molecule. The respective binding moieties may thus be brought into association by the target molecule in a binding complex. The target molecule may be any target molecule described herein, and the binding moiety(ies) any that provide for binding thereof. The binding moiety is typically comprised in the oxidoreductase enzyme such that binding of the target molecule can effect a conformational change in the enzyme,

[0093] As generally used herein a "binding moiety" or "binding moieties" refer to one or a plurality of molecules or biological or chemical components or entities that are capable of recognizing and/or binding

each other, and/or one or more other target molecules. Binding moieties may be proteins, nucleic acids (e.g. single-stranded or double-stranded DNA or RNA), sugars, oligosaccharides, polysaccharides or other carbohydrates, lipids or any combinations of these such as glycoproteins, PNA constructs etc or molecular components thereof. By way of example only, binding moieties may be, or comprise: (i) an amino acid sequence of a ligand binding domain of a receptor responsive to binding of a target molecule such as a cognate growth factor, cytokine, a hormone (e.g. insulin), neurotransmitters etc; (ii) an amino acid sequence of an ion or metabolite transporter capable of, or responsive to, binding of a target molecule such as an ion or metabolite (e.g. a Ca^{2+} -binding protein such as calmodulin or calcineurin or a glucose transporter); (iii) a zinc finger amino acid sequence responsive to zinc-dependent binding a DNA target molecule; (iv) a helix-loop-helix amino acid sequence responsive to binding a DNA target molecule; (v) a pleckstrin homology domain amino acid sequence responsive to binding of a phosphoinositide target molecule; (vi) an amino acid sequence of a Src homology 2- or Src homology 3-domain responsive to a signaling protein; (vii) an amino acid sequence of an antigen responsive to binding of an antibody target molecule; or (viii) an amino acid sequence of a protein kinase or phosphatase responsive to binding of a phosphorylatable or phosphorylated target molecule; (ix) ubiquitin-binding domains; (x) proteins or protein domains that bind small molecules, drugs or antibiotics such as rapamycin-binding FKBP and FRB domains; (xi) single- or double-stranded DNA, RNA or PNA constructs that bind nucleic acid target molecules, such as where the DNA or RNA are coupled or cross-linked to an amino acid sequence or other protein-nucleic acid interaction; and/or (xii) an affinity clamp such as a PDZ-FH3 domain fusion; inclusive of modified or engineered versions thereof, although without limitation thereto.

[0094] Particular binding moieties of use in the invention are provided by SEQ ID NOs 3-8, 13-14, 15-16, 18-19, 22-23 and 26-28 and variants thereof. Variants are typically functionally binding variants for the relevant respective binding moiety. Combinations of such binding moieties forming respective binding moieties are provided in the Examples and described further herein.

[0095] It will also be appreciated that binding moieties may be modified or chemically derivatized such as with binding agents such as biotin, avidin, epitope tags, lectins, carbohydrates, lipids although without limitation thereto.

[0096] In one embodiment, the binding moieties comprise an amino acid sequence of at least a fragment of any protein or protein fragment or domain that can bind or interact directly, or bind to a target molecule. The binding moiety may be, or comprise a protein such as a peptide, antibody, antibody fragment or any other protein scaffold that can be suitably engineered to create or comprise a binding portion, domain or region (e.g. reviewed in Binz et al., 2005 Nature Biotechnology, 23, 1257-68.) which binds a target molecule.

[0097] In one particular embodiment, the binding moieties respectively are, or comprise, amino acid sequences of an affinity clamp. The affinity clamp preferably comprises a recognition domain and, optionally, an enhancer domain. The recognition domain is typically capable of binding one or more target molecules, such as described in (i)-(ix) above. Recognition domains may include, but are not limited to, domains involved in phospho-tyrosine binding (e.g. SH2, PTB), phospho-serine binding (e.g. UIM, GAT, CUE, BTB/POZ, VHS, UBA, RING, HECT, WW, 14-3-3, Polo-box), phospho-threonine binding (e.g. FHA, WW, Polo-box), proline-rich region binding (e.g. EVH1, SH3, GYF), acetylated lysine binding (e.g. Bromo), methylated lysine binding (e.g. Chromo, PHD), apoptosis (e.g. BIR, TRAF, DED, Death, CARD, BH), cytoskeleton modulation (e.g. ADF, GEL, DH, CH, FH2), ubiquitin-binding domains or modified or engineered versions thereof, or other cellular functions (e.g. EH, CC, VHL, TUDOR, PUF Repeat, PAS, MH1, LRR1, IQ, HEAT, GRIP, TUBBY, SNARE, TPR, TIR, START, SOCS Box, SAM, RGS, PDZ, PB1, LIM, F-BOX, ENTH, EF-Hand, SHADOW, ARM, ANK).

[0098] The enhancer domain typically increases or enhances the binding affinity for at least one or the target molecules. In some embodiments, the affinity may be increased by at least 10, 100 or 1000 fold compared to that of the recognition domain alone. The affinity clamp may further comprise linker connecting the recognition domain and the enhancer domain.

[0099] In one particular embodiment, the affinity clamp comprises a recognition domain that comprises at least a portion or fragment of a PDZ domain and an enhancer domain that comprises at least a portion or fragment of a fibronectin type III domain. The PDZ domain may be derived from a human Erbin protein. Erbin-PDZ (ePDZ) binds to target molecules such as the C-termini of p120-related catenins (such as δ).

[0100] In some embodiments, the affinity clamp may comprise one or more connector amino acid sequences. For example, a connector amino acid sequence may connect the protease amino acid sequence (such as comprising a protease amino acid sequence) to the Erbin-PDZ domain, the Erbin-PDZ domain to the FN3 domain and/or the FN3 domain to the inhibitor.

[0102] The above discussion of affinity clamps and target molecules therefor also applies to selection of an affinity clamp as a heterologous amino acid sequence and selection of a binding peptide therefor.

[0104] In another embodiment, the binding moieties may be or comprise an antibody or antibody fragment, inclusive of monoclonal and polyclonal antibodies, recombinant antibodies, Fab and Fab'2 fragments, diabodies and single chain antibody fragments (e.g. scVs), although without limitation thereto. Suitably, the first and second binding moieties may be or comprise respective antibodies or antibody fragments that bind a target molecule.

[0106] The target molecule may be any ligand, analyte, small organic molecule, epitope, domain, fragment, subunit, moiety or combination thereof, such as a protein inclusive of antibodies and antibody fragments, antigens, enzymes, phosphoproteins, glycoproteins, lipoproteins and glycoproteins, lipid, phospholipids, carbohydrates inclusive of simple sugars, disaccharides and polysaccharides, nucleic acids, nucleoprotein or any other molecule or analyte. These include drugs and other pharmaceuticals including antibiotics, banned substances, illicit drugs or drugs of addiction, chemotherapeutic agents and lead compounds in drug design and screening, molecules and analytes typically found in biological samples such as biomarkers, tumour and other antigens, receptors, DNA-binding proteins inclusive of transcription factors, hormones, neurotransmitters, growth factors, cytokines, receptors, metabolic enzymes, signaling molecules, nucleic acids such as DNA and RNA, membrane lipids and other cellular components, pathogen-derived molecules inclusive of viral, bacterial, protozoan, fungal and worm proteins, lipids, carbohydrates and nucleic acids, although without limitation thereto. As previously, described, it will be appreciated that the "same" target molecule can be bound by different, respective binding moieties.

[0108] In some embodiments, the target molecule is a small organic molecule such as rapamycin. In such embodiments, the first and second binding moieties may be, respectively an FKBP and FRB. A preferred FKBP and FRB pair comprises the sequences of SEQ ID NOs: 13 and 14 or variants thereof.

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complex. Examples of these binding moieties are provided as SEQ ID NOs 13, 18 and 19 or a variant thereof. SEQ ID NO: 19 or a variant thereof may be fused with a calmodulin-binding peptide described herein such as SEQ ID NO: 8 or a variant thereof. Such a fusion protein may comprise SEQ ID NO: 16 or a variant thereof.

[0110] In some embodiments, the target molecule is a cytokine, such as IL-18. In such embodiments, the first and second binding moieties may be, respectively the cytokine and a cytokine-binding protein. Examples of such binding moieties are provided by the cytokine IL-18 (SEQ ID NO: 42) and IL-18 binding protein (SEQ ID NO: 41). An example of use of these binding moieties is provided by the dissociative sensor of SEQ ID NO: 37, incorporating an ePDZ domain and an IL-18 binding protein, and the fusion peptide of SEQ ID NO: 38, incorporating an ePDZ peptide and IL-18.

[0111] Particular oxidoreductase and peptide fusion molecules incorporating respective binding moieties as described above are provided as SEQ ID Nos 11-12, 16, 20-21, 24-25 and 29, and SEQ ID NOs 37-38 or variants thereof.

Autoinhibited Enzymes/Protease Activation

[0112] The above-described oxidoreductase (such as GDH) enzymes may comprise one or more protease cleavage sites, wherein cleavage of a said site by a protease displaces the inhibitory moiety to activate catalytic activity of the enzyme. The enzyme may further comprise a sequence enhancing binding and/or cleavage efficiency of the protease. An example of such a sequence enhancing thrombin binding is provided as SEQ ID NO: 36 or a variant thereof.

[0113] The oxidoreductase enzyme may comprise a binding moiety capable of interacting with a respective binding moiety on a further molecule, wherein interaction between the binding moieties displaces the inhibitory moiety to activate catalytic activity of the enzyme. Such an oxidoreductase enzyme may further comprise one or more protease cleavage sites, wherein the further molecule additionally comprises a protease and interaction between the binding moieties acts to bring the protease into proximity with a said site to cleave said site and displace the inhibitory moiety. The binding moieties and protease cleavage site(s) may be selected from any of those described herein. In an embodiment where the oxidoreductase enzyme displays a reduction in catalytic activity in the presence of binding of said peptide to the heterologous amino acid sequence, as described above, the oxidoreductase enzyme may comprise one or protease cleavage sites, where cleavage of a said site by a protease releases said peptide to thereby enhance catalytic activity of the enzyme. The protease may comprise a binding moiety for a respective binding moiety in said enzyme so as to bring it into proximity to the enzyme for cleavage of said site. The interaction between the binding moieties may be dependent on the presence of a target molecule.

[0114] A "protease" is a protein which displays, or is capable of displaying, an ability to hydrolyse or otherwise cleave a peptide bond. Like terms include "proteinase" and "peptidase". Proteases include serine proteases, cysteine proteases, metalloproteases, threonine proteases, aspartate proteases, glutamic acid proteases, acid proteases, neutral proteases, alkaline proteases, exoproteases, aminopeptidases and endopeptidases although without limitation thereto. Proteases may be purified or synthetic (e.g. recombinant synthetic) forms of naturally-occurring proteases or may be engineered or modified proteases which comprise one or more fragments or domains of naturally-occurring proteases which, optionally, have been further modified to possess one or more desired characteristics, activities or properties.

[0115] The target protease may be any protease for which a protease cleavage site is known. Suitably, the target protease is detectable in a biological sample obtainable from an organism, inclusive of bacteria, plants and animals. Animals may include humans and other mammals. Non-limiting examples of target proteases include proteases involved in blood coagulation such as thrombin, plasmin, factor VII, factor IX, factor X, factor Xa, factor XI, factor XII (Hageman factor) and other proteases such as kallikreins (e.g. kallikrein III, P-30 or prostate specific antigen), matrix metalloproteinases (such as involved in wounds and ulcers; e.g. MMP7 and MMP9), adamalysins, serralysins, astacins and other proteases of the metzincin superfamily, trypsin, chymotrypsin, elastase, cathepsin G, pepsin and carboxypeptidase A as well as proteases of pathogenic viruses such as HIV protease, West Nile NS3 protease and dengue virus protease although without limitation thereto.

[0117] In some embodiments, proteases are derived from, or encoded by, a viral genome. Typically, such proteases are dependent on expression and proteolytic processing of a polyprotein and/or other events required as part of the life cycle of viruses such as Picornavirales, Nidovirales, Herpesvirales, Retroviruses and Adenoviruses, although without limitation thereto. Particular examples of proteases include: Potyviridae proteases such as the NIa protease of tobacco etch virus (TEV), tobacco vein mottling virus (TVMV), sugarcane mosaic virus (SMV) etc; Flaviviridae proteases such as the NS3 protease of hepatitis C virus (HCV); Picornaviridae proteases such as the 3C protease of EV71, Norovirus etc, the 2A protease of human rhinovirus, coxsackievirus B4 etc and the leader protease of foot and mouth disease virus (FMDV) etc; Coronaviridae proteases such as the 3C-like protease of SARS-CoV, IBV-CoV and Herpesvirus proteases such as HSV-1, HSV-2, HCMV and MCMV proteases etc, although without limitation thereto.

[0119] In other embodiments, proteases are SUMO related proteases that includes ubiquitin (Ub), NEDD8, and Atg 8 proteases. These proteases are converted into an autoinhibited form by fusion with their respective recognition domains (e.g SUMO) via a protease-resistant linker.

[0121] Examples of autoinhibited sensors responsive to protease cleavage are provided by SEQ ID NOs 39 and 40 or variants thereof. These sensors incorporate an autoinhibitory module based on inhibitory interaction between an ePDZ domain and an ePDZ peptide, separated by a protease cleavage site. Protease cleavage disrupts the inhibitory interaction and thus provides for activation of the sensor.

[0123] It will be appreciated that the biosensors and the molecular components thereof described herein may be, or comprise, contiguous amino acid sequences such as in the form of chimeric proteins or fusion proteins as are well understood in the art. Optionally, respective amino acid sequences (e.g binding moieties, enzyme amino acid sequences, protease amino acid sequences etc) may be discrete or separate amino acid sequences linked or connected by spacers or linkers (e.g. amino acids, amino acid sequences, nucleotides, nucleotide sequences or other molecules) to optimize features or activities such as target molecule recognition, binding and enzyme activity or inhibition, although without limitation thereto. Non-limiting examples of amino acid sequences inclusive of enzyme amino acid sequences, engineered mutants, linkers, protease cleavage sites, and binding moieties are provided as sequences of the invention below, as SEQ ID NOS: 1-42.

<https://appft.uspto.gov/netacgi/nph-Parser?Sect1=PTO2&Sect2=HITOFF&p=1&u=%2Fnetacgi%2FPTO%2Fsearch-adv.html...> 17/42

[0125] The term "sequence identity" is used herein in its broadest sense to include the number of exact amino acid matches having regard to an appropriate alignment using a standard algorithm, having regard to the extent that sequences are identical over a window of comparison. Sequence identity may be determined using computer algorithms such as GAP, BESTFIT, FASTA and the BLAST family of programs as for example disclosed by Altschul et al., 1997, Nucl. Acids Res. 25 3389. A detailed discussion of sequence analysis can be found in Unit 19.3 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel et al. (John Wiley & Sons Inc NY, 1995-1999).

[0127] Polypeptides Representing First and Second Oxidoreductase Fragments

[0129] In particular, they have identified that PQQ-GDH of SEQ ID NO: 1 may be cleaved at a location comprising such residues to provide soluble polypeptide fragments, including at the preferred insert location described above. The inventors have identified in particular a soluble fragment representing residues 1-330 of SEQ ID NO:1, whose solubility is indicative of autonomous folding. Reconstitution of a stable enzyme from fragments can provide a further means of controlling enzyme activity, in addition to regulation by interaction between binding moieties and by interaction with a target molecule as described above.

[0131] The polypeptide comprising a first fragment sequence may be capable of reconstituting a stable catalytically active enzyme with said polypeptide comprising a second fragment sequence of said enzyme. In this embodiment, the polypeptide comprising a first fragment sequence of said enzyme is able to displace a corresponding fragment sequence of said enzyme which is engineered to maintain an enzyme in a

catalytically inactive state from a stable enzyme complex, to restore catalytic activity.

[0132] The polypeptide comprising a first fragment sequence may alternatively comprise one or more mutations which render a stable enzyme comprising said polypeptide catalytically inactive. In an embodiment relating to GDH, the respective amino acid sequences of the enzyme may be the sequences of SEQ ID NO: 30 or a variant thereof, and SEQ ID NO: 31 or a variant thereof. The "engineered mutant" typically comprises a H144 mutation and a mutation to one or more of Q76 and D143. The mutations are selected to reduce or abolish catalytic activity of the enzyme. Preferably, H144, Q76 and D143 are each mutated. These residues may be each mutated to alanine, or alternative mutations to alanine which reduce or abolish catalytic activity can be made. The engineered mutant may comprise the sequence of SEQ ID NO: 30 or a variant thereof, incorporating one or more or all of the above mutations, and which also produces a catalytically inactive enzyme when non-covalently associated with the at least one amino acid sequence of the enzyme. The variant may comprise alternative inactivating mutations to those discussed above at positions 76, 143 and 144. Such a polypeptide (also described as an engineered polypeptide) is also able to be displaced from said stable enzyme complex to restore catalytic activity.

[0133] Also provided is an oxidoreductase enzyme, preferably a GDH enzyme which comprises both a first fragment sequence which is engineered as described above, and also a said second fragment sequence as part of a contiguous polypeptide, where the first and second fragment sequences are separated by one or more protease cleavage sites, such that protease activity allows for the engineered fragment sequence to be displaced, and a first fragment sequence capable of restoring catalytic activity to then non-covalently associate with the second fragment sequence to form a stable catalytically active enzyme.

[0134] The polypeptides described above may comprise a binding moiety capable of interacting with a respective binding moiety on a counterpart polypeptide comprising a second fragment sequence of said enzyme, wherein the interaction between the binding moieties regulates catalytic activity of the reconstituted stable glucose dehydrogenase enzyme. The interaction between the binding moieties may be regulated by binding of a target molecule. The binding moieties and corresponding target molecule may be selected from any described herein.

[0135] A polypeptide described above may further comprises a sequence inhibiting interaction of the respective binding moieties, and one or more protease cleavage sites, wherein cleavage by the protease provides for interaction between the binding moieties. The polypeptide may further comprise a sequence enhancing binding and/or cleavage efficiency of the protease. The protease cleavage site and the sequence enhancing binding and/or cleavage efficiency of the protease may be selected from any described herein.

[0136] In particular embodiments, the first and second fragment sequences described above may be derived by cleavage of a GDH enzyme in a loop or turn region of a GDH enzyme corresponding to the loop connecting beta-sheets 4 and 5 of a PQQ-GDH or in a region corresponding to residues 328 to 332 of said enzyme (such as at residue 330 of SEQ ID NO:1). The skilled person is able to identify corresponding locations in other enzymes from structural analysis and sequence alignment. A corresponding location is typically one which allows for generation of functional fragments of said enzyme which are able to reconstitute a stable enzyme.

[0137] In this aspect, the invention additionally provides a method of engineering an oxidoreductase enzyme, preferably a glucose dehydrogenase (GDH) enzyme to provide first and second fragment sequences capable of reconstituting a stable enzyme. The method comprises selecting a suitable location in the enzyme comprising residues influencing substrate binding and at which the enzyme may be cleaved to provide said first and second fragment sequences. The method typically further comprises introducing mutations into one of said sequences which render a stable enzyme reconstituted from said sequence catalytically inactive. The method may further comprise adding one or more binding moieties to said sequences which assist non-covalent association of polypeptides comprising the sequences to reconstitute a stable catalytically active enzyme.

[0138] The invention further provides a polypeptide comprising a first fragment sequence of a GDH enzyme which comprises SEQ ID NO: 30 or a variant thereof. This polypeptide may be a polypeptide capable of reconstituting a stable catalytically active GDH enzyme as described above. This polypeptide may be engineered to render a stable enzyme comprising said polypeptide catalytically inactive as described above.

[0139] The invention further provides a polypeptide comprising a second fragment sequence of a GDH enzyme which comprises SEQ ID NO: 31 or a variant thereof. A variant of SEQ ID NO: 31 may be a sequence which when included in a said polypeptide is capable of reconstituting a stable GDH enzyme together with a polypeptide comprising SEQ ID NO: 30 as described above.

[0140] The above polypeptides comprising SEQ ID NO: 30 or a variant thereof and SEQ ID NO: 31 or a variant thereof may further comprise one or more binding moieties selected from any described herein. Typically, a binding moiety is provided C-terminal to the sequence of SEQ ID NO: 30 or variant thereof, and N-terminal to the sequence of SEQ ID NO: 31 or variant thereof in a said polypeptide.

[0142] Also provided is a GDH enzyme comprising the sequence of SEQ NO: 30 or a variant thereof, and further engineered to comprise catalytically inactivating mutations as described above, and additionally the sequence of SEQ ID NO: 31 or a variant thereof, wherein one or more protease cleavage sites are located between said sequences, such that cleavage by a protease is able to displace said polypeptide comprising the catalytically inactive sequence from said enzyme. The GDH enzyme may further comprise a binding moiety capable of interacting with a respective binding moiety on a polypeptide comprising a first fragment sequence of a GDH enzyme which comprises SEQ ID NO: 30 or a variant thereof, optionally in the presence of a target molecule, wherein interaction between the binding moieties allows for reconstitution of a stable GDH enzyme.

Biosensors

[0145] The invention also generally relates to a biosensor comprising an enzyme and a heterologous amino acid sequence that releasably maintains said enzyme in a catalytically inactive state in the presence of a peptide, wherein the heterologous amino acid sequence binds to the peptide to switch the enzyme from a catalytically active state to a catalytically inactive state. The inventors have surprisingly identified that, in contrast to biosensor architectures previously described in which catalytic activity of an enzyme is increased upon association with a target molecule, it is possible to provide a biosensor comprising a heterologous amino acid sequence and a binding peptide therefor in which interaction between the peptide and the enzyme

Other Aspects

[0147] A further aspect of the invention provides a method of detecting a target molecule, said method including the step of contacting the biosensor, oxidoreductase enzyme or polypeptides comprising first and second fragment sequences of any of the aforementioned aspects with a sample to thereby determine the presence or absence of the target molecule in the sample. Suitably, the sample is a biological sample. Biological samples may include organ samples, tissue samples, cellular samples, fluid samples or any other sample obtainable, obtained, derivable or derived from an organism or a component of the organism. The biological sample can comprise a fermentation medium, feedstock or food product such as for example, but not limited to, dairy products. In particular embodiments, the biological sample is obtainable from a mammal, preferably a human. By way of example, the biological sample may be a fluid sample such as blood, serum, plasma, urine, saliva, tears, sweat, cerebrospinal fluid or amniotic fluid, a tissue sample such as a tissue or organ biopsy or may be a cellular sample such as a sample comprising red blood cells, lymphocytes, tumour cells or skin cells, although without limitation thereto. A particular type of biological sample is a pathology sample.

[0148] Suitably, the enzyme activity of the biosensor is not substantially inhibited by components of the sample (e.g. serum proteins, metabolites, cells, cellular debris and components, naturally-occurring protease inhibitors etc).

[0149] In one embodiment, the biosensor and/or methods of use may be applicable to drug testing such as for detecting the use of illicit drugs of addiction (e.g cannabinoids, amphetamines, cocaine, heroin etc.) and/or for the detection of performance-enhancing substances in sport and/or masking agents that are typically used to avoid detection of performance-enhancing substances. This may be applicable to the detection of banned performance-enhancing substances in humans and/or other mammals such as racehorses and greyhounds that may be subjected to illicit "doping" to enhance performance.

[0150] A yet further aspect of the invention provides a method of diagnosis of a disease or condition in an organism, said method including the step of contacting the biosensor, oxidoreductase enzyme or polypeptides comprising first and second fragment sequences of any of the aforementioned aspects with a biological sample obtained from the organism to thereby determine the presence or absence of a target molecule in the biological sample, determination of the presence or absence of the target molecule facilitating diagnosis of the disease or condition. The organism may include plants and animals inclusive of fish, avians and mammals such as humans. Preferably the organism is a human. The disease or condition may be any where detection of a target molecule assists diagnosis. Non limiting examples of target molecules or analytes include blood coagulation factors such as previously described, kallikreins inclusive of PSA, matrix metalloproteinases, viral and bacterial proteases, antibodies, glucose, triglycerides, lipoproteins, cholesterol, tumour antigens, lymphocyte antigens, autoantigens and autoantibodies, drugs, salts, creatinine, blood serum or plasma proteins, pesticides, uric acid, products and intermediates of human and animal metabolism and metals. This preferred aspect of the invention may be adapted to be performed as a "point of care" method whereby determination of the presence or absence of the target molecule may occur at a patient location which is then either analysed at that location or transmitted to a remote location for diagnosis of the disease or condition.

[0151] Diagnostic aspects of the invention may also be in the form of a kit comprising one or a plurality of different biosensors capable of detecting one or a plurality of different target molecules. In this regard, a kit

[0152] A still yet further aspect of the invention provides a detection device that comprises a cell or chamber that comprises the biosensor, oxidoreductase enzyme or polypeptides comprising first and second fragment sequences of any of the aforementioned aspects. Suitably, a sample may be introduced into the cell or chamber to thereby facilitate detection of a target molecule. In certain embodiments, the detection device is capable of providing an electrochemical, acoustic and/or optical signal that indicates the presence of the target molecule.

[0154] The detection device may further provide a disease diagnosis from a diagnostic target result by comprising: a processor; and

[0156] perform a set of functions including: analysing a diagnostic test result and providing a diagnosis of the disease or condition.

[0157] The detection device may further provide for communicating a diagnostic test result by comprising: a processor; and

[0158] a memory coupled to the processor, the memory including computer readable program code components that, when executed by the processor, perform a set of functions including: transmitting a diagnostic result to a receiving device; and optionally receiving a diagnosis of the disease or condition from the or another receiving device.

[0159] The biosensor, oxidoreductase enzyme or polypeptides comprising first and second fragment sequences of any of the aspects described herein may form part of a biofuel cell. The biofuel cells may comprise the biosensor, oxidoreductase enzyme or polypeptides comprising first and second fragment sequences of any of the aspects described herein located at the anode. As described herein the biosensor or enzyme may act as an electron donor and the electrons may flow from the anode to the cathode in the biofuel cell. The biosensor or enzyme may thereby provide electrons for a chemical reaction occurring at the cathode of the biofuel cell.

[0160] The invention further provides a nucleic acid (typically in isolated form) encoding the biosensor of any of the aforementioned aspects, or any component thereof, including an oxidoreductase enzyme of the invention or a polypeptide comprising a first or second fragment sequence of the invention. The nucleic acid may encode any of SEQ ID Nos 3-12, 16, 20-21, 24-25, 27-31 and 37-40 or a variant thereof as discussed above. Another related aspect of the invention provides a genetic construct comprising the isolated nucleic acid of the aforementioned aspect. A further related aspect of the invention provides a host cell comprising the genetic construct of the aforementioned aspect. The term "nucleic acid" as used herein designates single- or double-stranded mRNA, RNA, cRNA, RNAi, siRNA and DNA inclusive of cDNA, mitochondrial DNA (mtDNA) and genomic DNA. The invention also provides variants and/or fragments of the isolated nucleic acids. Variants may comprise a nucleotide sequence at least 70%, at least 75%, preferably at least 80%, at least 85%, more preferably at least 90%, 91%, 93%, 94%, 95%, 96%, 97%, 98% or 99% nucleotide sequence identity with any nucleotide sequence disclosed herein. In other embodiments, nucleic acid variants may hybridize with the nucleotide sequence of with any nucleotide sequence disclosed herein, under high stringency conditions. Fragments may comprise or consist of up to 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95-99% of the contiguous nucleotides present in any nucleotide sequence disclosed herein. Fragments may comprise or consist of up to 20, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900 950, 1000, 1050, 1100, 1150, 1200, 1350 or 1300 contiguous nucleotides present in any nucleotide sequence disclosed herein.

[0162] As generally used herein, a "genetic construct" is an artificially created nucleic acid that incorporates, and/or facilitates use of, an isolated nucleic acid disclosed herein.

[0164] The one or more additional nucleotide sequences are typically regulatory nucleotide sequences. By "operably linked" or "operably connected" is meant that said regulatory nucleotide sequence(s) is/are positioned relative to the nucleic acid to be expressed to initiate, regulate or otherwise control expression of the nucleic acid.

[0166] Suitable host cells for expression may be prokaryotic or eukaryotic, such as bacterial cells inclusive of *Escherichia coli* (DH5a for example), yeast cells such as *S. cerevisiae* or *Pichia pastoris*, insect cells such as SF9 cells utilized with a baculovirus expression system, or any of various mammalian or other animal host cells such as CHO, BHK or 293 cells, although without limitation thereto. Introduction of expression constructs into suitable host cells may be by way of techniques including but not limited to electroporation, heat shock, calcium phosphate precipitation, DEAE dextran-mediated transfection, liposome-based transfection (e.g. lipofectin, lipofectamine), protoplast fusion, microinjection or microparticle bombardment, as are well known in the art.

[0168] The invention additionally provides a method of engineering an oxidoreductase enzyme, preferably a glucose dehydrogenase (GDH) enzyme comprising a heterologous amino acid sequence which is responsive to a peptide, wherein binding of the peptide to the heterologous amino acid sequence reversibly regulates catalytic activity of the enzyme. The method comprises selecting a suitable location in the enzyme able to tolerate insertion of the heterologous amino acid sequence, typically a location comprising residues influencing substrate binding by and/or catalytic activity of said enzyme as described above, and inserting said heterologous amino acid sequence into the enzyme, such that an enzyme is engineered which responds

[0169] So that the invention may be readily understood and put into practical effect, embodiments of the invention will be described with reference to the following non-limiting Examples.

Materials and Methods

[0170] The constructs of GDH-CaM or GDH-PDZ chimeric proteins were generated by Gibson Assembly.TM. method according the manufacturer instruction (New England Biolab) and cloned into PET28a vector. The gene fragments for the assemble were made either by PCR or by Gblcok gene synthesis from IDT (Integrated DNA Technologies). The protein expression and purification were described by Olsthoorn & Duine.sup.19. The purified GDH-CaM were reconstituted by adding PQQ with 1:1.5 ratio. This ratio for reconstitution of GDH and PQQ was also used in all other experiments using PQQ-GDH enzymes described herein.

Analysis of GDH Enzymatic Activity

[0172] The GDH enzyme assay was performed as described by Yu et al.^{sup.20} Briefly, the 1.5-mL assay system consisted of 20 mM glucose, 0.6 mM phenazine methosulfate, 0.06 mM 2,6-dichlorophenol, 10 mM MOPS (pH 7.0), and corresponding concentration of CaCl₂ and enzyme. The enzymatic assay was performed at 25.degree. C. by monitoring the reduction in the absorbance of 2,6-dichlorophenol at 600 nm.

[0173] We previously identified the loop connecting strands A and B of beta-sheet 3 of PQQ-GDH as a site able to tolerate insertion of a calmodulin protein. The resulting calmodulin-GDH chimera (FIG. 1A) demonstrated calcium-binding activity and acted solely as a calcium sensor. The same location was also amenable to splitting GDH into two inactive fragments that could then be reconstituted into an active enzyme via scaffolding interactions with an analyte (FIG. 1B, SEQ ID NOs 43 and 44). The splitting approach allowed for coupling of GDH activity to detection of various analytes, but required proteolytic cleavage and purification of components, and also had a response rate governed by the rate of reconstitution of components. Analyte-driven dimerisation was also required for activity.

[0175] We conjectured that dislocation of either of these residues will impact the catalysis and if carried out in a reversible fashion could be utilized for controlling enzyme activity. To test this idea we inserted calmodulin (CaM) domain into GDH at the position 330 and produced the resulting CaM-GDH chimeric protein (SEQ ID NO: 10) in recombinant form. The resulting protein displayed some GDH activity that was potentially suppressed by addition of Ca^{2+} (FIG. 2A). However, addition of calmodulin binding peptide (CaM-BP, SEQ ID NO: 4) extracted from crystal structure of CaM:CBP complex (PDB:2BBM) increased the GDH activity of the chimera in a dose dependent fashion. The activity of fully activated fusion represented 50% of activity of the wild-type protein. Fit of the observed reaction rates showed that peptide bound to the enzyme with the affinity of 11 nM indicating that binding affinity was decreased due to the by the

Example 2-Construction of Two Component Biosensors Based on the Peptide-Activated GDH Chimera

Example 4--Developing an OFF GDH-Based Biosensor

[0187] The GDH-based OFF switch can also be converted into an autoinhibited protease biosensor, activatable by protease cleavage. This module is shown in FIG. 6A, with activity data for different variants following protease cleavage as shown in FIGS. 6B-6D. FIG. 6E illustrates how the autoinhibited protease biosensor could be subsequently integrated in to the ultrasensitive two component architecture.

Example 5--Comparison of Sensitivity of New Architecture with Previous Split Architecture

[0189] The data shown in FIG. 7 demonstrated that the insert biosensor had both faster rate of response and higher total electron yield. Therefore use of a GDH-CaM chimera-based biosensor is improved over the split architecture. Furthermore preparation of the GDH-CaM chimera-based biosensor did not require proteolytic cleavage of the precursor protein making their preparation straightforward.

[0190] Throughout the specification, the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. Various changes and modifications may be made to the embodiments described and illustrated without departing from the present invention.

[0191] The disclosure of each patent and scientific document, computer program and algorithm referred to in

TABLE-US-00001 SEQUENCES OF THE INVENTION mature PQQ-GDH protein (SEQ ID NO: 1)
DVPLIPSQFAKAKSENFDDKKVILSNLNKPHALLWGPDNQIWLTERATGKILRVN
PESGSVKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKE
LPNQTIIRRYTYNKSTDITLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQ
GRNQLAYLFLPNQAQHTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNG
VVSHIYTLGHRNPQGLAFTPNGKLLQSEQGPNSDDEINLIVKGGNYGWPNAVAG
YKDDSGYAYANYSAANKTIKDLAQNGVKVAAGVPVTKESEWTGKNFVPL
KTLYTVQDTYNYNDPTCGEMTYICWPTVAPSSAYVYKGGKKAITGWENTLLV
PSLKRGVIFRIKLDPTYSTTYDDAVPMFKSNRNRDVIASPDGNVLYVLTDTAG
NVQKDDGSVTNTLENPGSLIKFTYKAK Protein sequence before cleavage of signal sequence (SEQ ID
NO: 2) MNKHLLAKIALLGAAQLVTLAFAADVPLIPSQFAKAKSENFDDKKVILSNLNKPH
ALLWGPDNQIWLTERATGKILRVNPESGSVKTVFQVPEIVNDADGQNGLLGFA
FHPDFKNNPYIYISGTFKNPKSTDKEKLPNQTIIRRYTYNKSTDITLEKPVDLLAGLP
SSKDHQSGRLVIGPDQKIYYTIGDQGRNQLAYLFLPNQAQHTPTQQELNGKDY
HTYMGKVLRLNLDGSIPKDNPSFNGVVSHIYTLGHRNPQGLAFTPNGKLLQSEQ
GPNSDDEINLIVKGGNYGWPNAVAGYKDDSGYAYANYSAANKTIKDLAQNGV
KVAAGVPVTKESEWTGKNFVPLKTLYTVQDTYNYNDPTCGEMTYICWPTVA
PSSAYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTYSTTYDDAVPMFKSN
RNRDVIASPDGNVLYVLTDTAGNVQKDDGSVTNTLENPGSLIKFTYKAK Calmodulin protein (SEQ
ID NO: 3) TEEQIAEFKEAFSLFDKDGDTITTKELGTVMRSLGQNPTEAELQDMINEVDAD
GNGTIDFPEFLTMMARKMKDTSDEEIREAFRVFDKDGNGYISAAELRHVMTN
LGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTA CaM-binding peptide from structure
PDB:2BBM (SEQ ID NO: 4) KRRWKKNFIAVSAANRFFKISSSGAL Modified CaM-BPs
KRRWKKNFIAVSAANRFFKIS (SEQ ID NO: 5) KRRWKKNFIAVSAANR (SEQ ID NO: 6)
KRRWKKNFIA (SEQ ID NO: 7) Preferred Modified CaM-BP KRRWKKNFIAVASASA (SEQ ID NO: 8)
GDH-CaM (first generation) (SEQ ID NO: 9)

DVPLIPSQFAKAKSENFDKKVILSNLNKPHALLWGPNDQIWLTERATGKILRVN
PESGSVKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKE
LPNQTIIRRYTYNKSTDTLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQ
GRNQLAYLFLPNQAQHTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNG
VVSHIYTLGHRNPQGLAFTPNGKLLQSEQGPSNDDEINLIVKGGNYGWPNAVAG
YKDDSGYAYANYSAANKTIKDLAQNGVKVAAGVPVTKESEWTGKNFVPPL
KTLYTVQDGS GSGSGTEEQIAEFKEAFSLFDKDGDTITTTELGTVMRSLGQN
PTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTESEEEIREAFRVFDKD
GNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTA
GYNYNDPTCGEMTYICWPTVAPSSAYVYKGGKKAITGWENTLLVPSLKRGVIF
RIKLDPTYSTTYDDAVPMFKSNNRYRDVIASPDGNVLYVLTDAGNVQKDDGS
VTNTLENPGSLIKFTYKAKHHHHHH GDH-CaM (second generation) (SEQ ID NO: 10)
DVPLIPSQFAKAKSENFDKKVILSNLNKPHALLWGPNDQIWLTERATGKILRVN
PESGSVKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKE
LPNQTIIRRYTYNKSTDTLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQ
GRNQLAYLFLPNQAQHTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNG
VVSHIYTLGHRNPQGLAFTPNGKLLQSEQGPSNDDEINLIVKGGNYGWPNAVAG
YKDDSGYAYANYSAANKTIKDLAQNGVKVAAGVPVTKESEWTGKNFVPPL
KTLYTVQDGS GSGTEEQIAEFKEAFSLFDKDGDTITTTELGTVMRSLGQNPT
EAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTESEEEIREAFRVFDKDGNGY
ISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAGGSG
GYNYNDPTCGEMTYICWPTVAPSSAYVYKGGKKAITGWENTLLVPSLKRGVIF
RIKLDPTYSTTYDDAVPMFKSNNRYRDVIASPDGNVLYVLTDAGNVQKDDGS
VTNTLENPGSLIKFTYKAKHHHHHH GDH-CaM-FKBP12 (SEQ ID NO: 11)
DVPLIPSQFAKAKSENFDKKVILSNLNKPHALLWGPNDQIWLTERATGKILRVN
PESGSVKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKE
LPNQTIIRRYTYNKSTDTLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQ
GRNQLAYLFLPNQAQHTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNG
VVSHIYTLGHRNPQGLAFTPNGKLLQSEQGPSNDDEINLIVKGGNYGWPNAVAG
YKDDSGYAYANYSAANKTIKDLAONGVKVAAGVPVTKESEWTGKNFVPPL

<https://appft.uspto.gov/netacgi/nph-Parser?Sect1=PTO2&Sect2=HITOFF&p=1&u=%2Fnetacgi%2FPTO%2Fsearch-adv.html...> 28/42

DVPLIPSOFAKAKSENFDDKKVILSNLNKPHALLWGPDNOIWLTERATGKILRVN

Human IL18 dissociative sensor (SEQ ID NO: 37) (GDH-ePDZ-Interleukin 18 binding protein)
DVPLIPSQFAKAKSENFDKKVILSNLNKPHALLWGPDNQIWLTERATGKILRVN
PESGSVKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKE
LPNQTIIRRYTYNKSTDTLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQ
GRNQLAYLFLPNQAQHTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNG
VVSHIYTLGHRNPQGLAFTPNGKLLQSEQGPNSDDEINLIVKGGNYGWPNAVAG
YKDDSGYAYANYSAANKTIKDLAQNGVKVAAGVPVTKESEWTGKNFVPPL
KTLYTVQDEDAPESGSPELGFSISGGVGGRGNPFRPDDDGIFVTRVQPEGPASKL
LQPGDKIIQANGYSFINIEHGQAVSLLKTFQNTVELIIVREVGNGAKQEIRVRVE
KDGGSGGVSSVPTNLEVVAATPTSLLISWDAYRELPSYYRITYGETGGNSPVQ
EFTVPGSKSTATISGLKPGVDYTITVYAHYNYHYYSSPISINYRSGSGPGYNYNDP
TCGEMTYICWPTVAPSSAYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTY
STTYDDAVPMFKSNNRYRDVIASPDGNVLYVLTDTAGNVQKDDGSVTNTLEN
PGSLIKFTYKAKGSGSGSGSGSGGGAMVETKCPNLDIVTSSGEFHCSGCVEHMP
EFSYMYWLAKDMKSDEDTKFIEHLGDGINEDETVRTTDGGITT LRKVLHVTDT
NKFAHYRFTCVLTTLDGVSCKNIWLKKLAAALEHHHHHHH Interleukin-18-ePDZ peptide (SEQ ID NO:
38) DGHHHHHHHGSGGYFGKLESKLSVIRNLNDQVLFIDQGNRPLFEDMTDSDSRDN
APRTIFIISMYKDSQPRGMAVTISVKSEKISTLSENKIISFKEMNPPDNIKDTKSDI
IFFQRSVPGHDNKMQFESSYEGYFLASEKERDLFKLILKKEDELGDRSIMFTVQ
NEDGSGSGSGSGSGSGGRGSIDTWV Auto-inhibited GDH-ePDZ peptide sensor-strong peptide (SEQ ID
NO: 39) (GDH-ePDZ-TVMV site-strong Peptide)
DVPLIPSQFAKAKSENFDKKVILSNLNKPHALLWGPDNQIWLTERATGKILRVN
PESGSVKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKE
LPNQTIIRRYTYNKSTDTLEKPVDLLAGLPSSKDHQSGRLVIGPDQKIYYTIGDQ
GRNQLAYLFLPNQAQHTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNG
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YKDDSGYAYANYSAANKTIKDLAQNGVKVAAGVPVTKESEWTGKNFVPPL
KTLYTVQDEDAPESGSPELGFSISGGVGGRGNPFRPDDDGIFVTRVQPEGPASKL
LQPGDKIIQANGYSFINIEHGQAVSLLKTFQNTVELIIVREVGNGAKQEIRVRVE
KDGGSGGVSSVPTNLEVVAATPTSLLISWDAYRELPSYYRITYGETGGNSPVQ
EFTVPGSKSTATISGLKPGVDYTITVYAHYNYHYYSSPISINYRSGSGPGYNYNDP
TCGEMTYICWPTVAPSSAYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTY
STTYDDAVPMFKSNNRYRDVIASPDGNVLYVLTDTAGNVQKDDGSVTNTLEN
PGSLIKFTYKAKGSGHHHHHHHSGGETVRFQSSGSGGRGSIDTWV Auto-inhibited GDH-ePDZ peptide
sensor-weak peptide (SEQ ID NO: 40) (GDH-ePDZ-TVMV site-weak Peptide)
DVPLIPSQFAKAKSENFDKKVILSNLNKPHALLWGPDNQIWLTERATGKILRVN
PESGSVKTVFQVPEIVNDADGQNGLLGFAFHPDFKNNPYIYISGTFKNPKSTDKE
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GRNQLAYLFLPNQAQHTPTQQELNGKDYHTYMGKVLRLNLDGSIPKDNPSFNG
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YKDDSGYAYANYSAANKTIKDLAQNGVKVAAGVPVTKESEWTGKNFVPPL
KTLYTVQDEDAPESGSPELGFSISGGVGGRGNPFRPDDDGIFVTRVQPEGPASKL
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KDGGSGGVSSVPTNLEVVAATPTSLLISWDAYRELPSYYRITYGETGGNSPVQ
EFTVPGSKSTATISGLKPGVDYTITVYAHYNYHYYSSPISINYRSGSGPGYNYNDP
TCGEMTYICWPTVAPSSAYVYKGGKKAITGWENTLLVPSLKRGVIFRIKLDPTY

REFERENCES

Sequence CWU 1

Ser Gly Ser Val Lys Thr Val Phe 50 55 60Gln Val Pro Glu Ile Val Asn Asp Ala Asp Gly Gln Asn Gly Leu
Leu65 70 75 80Gly Phe Ala Phe His Pro Asp Phe Lys Asn Asn Pro Tyr Ile Tyr Ile 85 90 95Ser Gly Thr Phe
Lys Asn Pro Lys Ser Thr Asp Lys Glu Leu Pro Asn 100 105 110Gln Thr Ile Ile Arg Arg Tyr Thr Tyr Asn Lys
Ser Thr Asp Thr Leu 115 120 125Glu Lys Pro Val Asp Leu Leu Ala Gly Leu Pro Ser Ser Lys Asp His 130
135 140Gln Ser Gly Arg Leu Val Ile Gly Pro Asp Gln Lys Ile Tyr Tyr Thr145 150 155 160Ile Gly Asp Gln
Gly Arg Asn Gln Leu Ala Tyr Leu Phe Leu Pro Asn 165 170 175Gln Ala Gln His Thr Pro Thr Gln Gln Glu
Leu Asn Gly Lys Asp Tyr 180 185 190His Thr Tyr Met Gly Lys Val Leu Arg Leu Asn Leu Asp Gly Ser Ile
195 200 205Pro Lys Asp Asn Pro Ser Phe Asn Gly Val Val Ser His Ile Tyr Thr 210 215 220Leu Gly His Arg
Asn Pro Gln Gly Leu Ala Phe Thr Pro Asn Gly Lys225 230 235 240Leu Leu Gln Ser Glu Gln Gly Pro Asn
Ser Asp Asp Glu Ile Asn Leu 245 250 255Ile Val Lys Gly Gly Asn Tyr Gly Trp Pro Asn Val Ala Gly Tyr Lys
260 265 270Asp Asp Ser Gly Tyr Ala Tyr Ala Asn Tyr Ser Ala Ala Ala Asn Lys 275 280 285Thr Ile Lys Asp
Leu Ala Gln Asn Gly Val Lys Val Ala Ala Gly Val 290 295 300Pro Val Thr Lys Glu Ser Glu Trp Thr Gly Lys
Asn Phe Val Pro Pro305 310 315 320Leu Lys Thr Leu Tyr Thr Val Gln Asp Thr Tyr Asn Tyr Asn Asp Pro
325 330 335Thr Cys Gly Glu Met Thr Tyr Ile Cys Trp Pro Thr Val Ala Pro Ser 340 345 350Ser Ala Tyr Val
Tyr Lys Gly Gly Lys Lys Ala Ile Thr Gly Trp Glu 355 360 365Asn Thr Leu Leu Val Pro Ser Leu Lys Arg
Gly Val Ile Phe Arg Ile 370 375 380Lys Leu Asp Pro Thr Tyr Ser Thr Thr Tyr Asp Asp Ala Val Pro Met385
390 395 400Phe Lys Ser Asn Asn Arg Tyr Arg Asp Val Ile Ala Ser Pro Asp Gly 405 410 415Asn Val Leu
Tyr Val Leu Thr Asp Thr Ala Gly Asn Val Gln Lys Asp 420 425 430Asp Gly Ser Val Thr Asn Thr Leu Glu
Asn Pro Gly Ser Leu Ile Lys 435 440 445Phe Thr Tyr Lys Ala Lys 4502478PRTArtificial SequenceProtein
sequence before cleavage of signal sequence 2Met Asn Lys His Leu Leu Ala Lys Ile Ala Leu Leu Gly Ala
Ala Gln1 5 10 15Leu Val Thr Leu Ser Ala Phe Ala Asp Val Pro Leu Ile Pro Ser Gln 20 25 30Phe Ala Lys Ala
Lys Ser Glu Asn Phe Asp Lys Lys Val Ile Leu Ser 35 40 45Asn Leu Asn Lys Pro His Ala Leu Leu Trp Gly
Pro Asp Asn Gln Ile 50 55 60Trp Leu Thr Glu Arg Ala Thr Gly Lys Ile Leu Arg Val Asn Pro Glu65 70 75
80Ser Gly Ser Val Lys Thr Val Phe Gln Val Pro Glu Ile Val Asn Asp 85 90 95Ala Asp Gly Gln Asn Gly Leu
Leu Gly Phe Ala Phe His Pro Asp Phe 100 105 110Lys Asn Asn Pro Tyr Ile Tyr Ile Ser Gly Thr Phe Lys Asn
Pro Lys 115 120 125Ser Thr Asp Lys Glu Leu Pro Asn Gln Thr Ile Ile Arg Arg Tyr Thr 130 135 140Tyr Asn
Lys Ser Thr Asp Thr Leu Glu Lys Pro Val Asp Leu Leu Ala145 150 155 160Gly Leu Pro Ser Ser Lys Asp
His Gln Ser Gly Arg Leu Val Ile Gly 165 170 175Pro Asp Gln Lys Ile Tyr Tyr Thr Ile Gly Asp Gln Gly Arg
Asn Gln 180 185 190Leu Ala Tyr Leu Phe Leu Pro Asn Gln Ala Gln His Thr Pro Thr Gln 195 200 205Gln
Glu Leu Asn Gly Lys Asp Tyr His Thr Tyr Met Gly Lys Val Leu 210 215 220Arg Leu Asn Leu Asp Gly Ser
Ile Pro Lys Asp Asn Pro Ser Phe Asn225 230 235 240Gly Val Val Ser His Ile Tyr Thr Leu Gly His Arg Asn
Pro Gln Gly 245 250 255Leu Ala Phe Thr Pro Asn Gly Lys Leu Leu Gln Ser Glu Gln Gly Pro 260 265
270Asn Ser Asp Asp Glu Ile Asn Leu Ile Val Lys Gly Gly Asn Tyr Gly 275 280 285Trp Pro Asn Val Ala Gly
Tyr Lys Asp Asp Ser Gly Tyr Ala Tyr Ala 290 295 300Asn Tyr Ser Ala Ala Ala Asn Lys Thr Ile Lys Asp
Leu Ala Gln Asn305 310 315 320Gly Val Lys Val Ala Ala Gly Val Pro Val Thr Lys Glu Ser Glu Trp 325 330
335Thr Gly Lys Asn Phe Val Pro Pro Leu Lys Thr Leu Tyr Thr Val Gln 340 345 350Asp Thr Tyr Asn Tyr
Asn Asp Pro Thr Cys Gly Glu Met Thr Tyr Ile 355 360 365Cys Trp Pro Thr Val Ala Pro Ser Ser Ala Tyr Val
Tyr Lys Gly Gly 370 375 380Lys Lys Ala Ile Thr Gly Trp Glu Asn Thr Leu Leu Val Pro Ser Leu385 390 395
400Lys Arg Gly Val Ile Phe Arg Ile Lys Leu Asp Pro Thr Tyr Ser Thr 405 410 415Thr Tyr Asp Asp Ala Val
Pro Met Phe Lys Ser Asn Asn Arg Tyr Arg 420 425 430Asp Val Ile Ala Ser Pro Asp Gly Asn Val Leu Tyr
Val Leu Thr Asp 435 440 445Thr Ala Gly Asn Val Gln Lys Asp Asp Gly Ser Val Thr Asn Thr Leu 450 455
460Glu Asn Pro Gly Ser Leu Ile Lys Phe Thr Tyr Lys Ala Lys465 470 4753143PRTArtificial
SequenceCalmodulin protein 3Thr Glu Glu Gln Ile Ala Glu Phe Lys Glu Ala Phe Ser Leu Phe Asp1 5 10
15Lys Asp Gly Asp Gly Thr Ile Thr Thr Lys Glu Leu Gly Thr Val Met 20 25 30Arg Ser Leu Gly Gln Asn
Pro Thr Glu Ala Glu Leu Gln Asp Met Ile 35 40 45Asn Glu Val Asp Ala Asp Gly Asn Gly Thr Ile Asp Phe
Pro Glu Phe 50 55 60Leu Thr Met Met Ala Arg Lys Met Lys Asp Thr Asp Ser Glu Glu Glu65 70 75 80Ile
Arg Glu Ala Phe Arg Val Phe Asp Lys Asp Gly Asn Gly Tyr Ile 85 90 95Ser Ala Ala Glu Leu Arg His Val
Met Thr Asn Leu Gly Glu Lys Leu 100 105 110Thr Asp Glu Glu Val Asp Glu Met Ile Arg Glu Ala Asp Ile
Asp Gly 115 120 125Asp Gly Gln Val Asn Tyr Glu Glu Phe Val Gln Met Met Thr Ala 130 135
140426PRTArtificial SequenceCaM-binding peptide from structure PDB2BBM 4Lys Arg Arg Trp Lys Lys
Asn Phe Ile Ala Val Ser Ala Ala Asn Arg1 5 10 15Phe Lys Lys Ile Ser Ser Ser Gly Ala Leu 20
25521PRTArtificial SequenceModified CaM-BP 5Lys Arg Arg Trp Lys Lys Asn Phe Ile Ala Val Ser Ala Ala
Asn Arg1 5 10 15Phe Lys Lys Ile Ser 20616PRTArtificial SequenceModified CaM-BP 6Lys Arg Arg Trp Lys
Lys Asn Phe Ile Ala Val Ser Ala Ala Asn Arg1 5 10 15710PRTArtificial SequenceModified CaM-BP 7Lys
Arg Arg Trp Lys Lys Asn Phe Ile Ala1 5 10816PRTArtificial SequencePreferred Modified CaM-BP 8Lys
Arg Arg Trp Lys Lys Asn Phe Ile Ala Val Ala Ser Ala Ser Ala1 5 10 159611PRTArtificial SequenceGDH-
CaM (first generation) 9Asp Val Pro Leu Ile Pro Ser Gln Phe Ala Lys Ala Lys Ser Glu Asn1 5 10 15Phe Asp
Lys Lys Val Ile Leu Ser Asn Leu Asn Lys Pro His Ala Leu 20 25 30Leu Trp Gly Pro Asp Asn Gln Ile Trp

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Leu Glu Glu Ser Val Ala Leu Arg Ile Ile Thr Glu Gly 165 170 175Ala Ser Ile Leu Arg Gln Glu Lys Asn Leu
Leu Asp Ile Asp Ala Pro 180 185 190Val Thr Val Cys Gly Asp Ile His Gly Gln Phe Phe Asp Leu Met Lys
195 200 205Leu Phe Glu Val Gly Gly Ser Pro Ala Asn Thr Arg Tyr Leu Phe Leu 210 215 220Gly Asp Tyr
Val Asp Arg Gly Tyr Phe Ser Ile Glu Cys Val Leu Tyr225 230 235 240Leu Trp Ala Leu Lys Ile Leu Tyr Pro
Lys Thr Leu Phe Leu Leu Arg 245 250 255Gly Asn His Glu Cys Arg His Leu Thr Glu Tyr Phe Thr Phe Lys
Gln 260 265 270Glu Cys Lys Ile Lys Tyr Ser Glu Arg Val Tyr Asp Ala Cys Met Asp 275 280 285Ala Phe
Asp Cys Leu Pro Leu Ala Ala Leu Met Asn Gln Gln Phe Leu 290 295 300Cys Val His Gly Gly Leu Ser Pro
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Met Cys 325 330 335Asp Ile Leu Trp Ser Asp Pro Leu Glu Asp Phe Gly Asn Glu Lys Thr 340 345 350Gln
Glu His Phe Thr His Asn Thr Val Arg Gly Cys Ser Tyr Phe Tyr 355 360 365Ser Tyr Pro Ala Val Cys Glu Phe
Leu Gln His Asn Asn Leu Leu Ser 370 375 380Ile Leu Arg Ala His Glu Ala Gln Asp Ala Gly Tyr Arg Met
Tyr Arg385 390 395 400Lys Ser Gln Thr Thr Gly Phe Pro Ser Leu Ile Thr Ile Phe Ser Ala 405 410 415Pro
Asn Tyr Leu Asp Val Tyr Asn Asn Lys Ala Ala Val Leu Lys Tyr 420 425 430Glu Asn Asn Val Met Asn Ile
Arg Gln Phe Asn Cys Ser Pro His Pro 435 440 445Tyr Trp Leu Pro Asn Phe Met Asp Val Phe Thr Trp Ser
Leu Pro Phe 450 455 460Val Gly Glu Lys Val Thr Glu Met Leu Val Asn Val Leu Asn Ile Cys465 470 475
480Ser Asp Asp Glu Leu Gly Ser Glu Glu Asp Gly Phe Asp Gly Ala Thr 485 490 495Ala Ala Ala Arg Leu
Val Thr Ala Gly Leu Val Leu Ala 500 50516209PRTArtificial SequenceCN beta subunit-CalM peptide
16Asp Gly His His His His His His Gly Gly Asn Glu Ala Ser Tyr Pro1 5 10 15Leu Glu Met Cys Ser His Phe
Asp Ala Asp Glu Ile Lys Arg Leu Gly 20 25 30Lys Arg Phe Lys Lys Leu Asp Leu Asp Asn Ser Gly Ser Leu
Ser Val 35 40 45Glu Glu Phe Met Ser Leu Pro Glu Leu Gln Gln Asn Pro Leu Val Gln 50 55 60Arg Val Ile
Asp Ile Phe Asp Thr Asp Gly Asn Gly Glu Val Asp Phe65 70 75 80Lys Glu Phe Ile Glu Gly Val Ser Gln Phe
Ser Val Lys Gly Asp Lys 85 90 95Glu Gln Lys Leu Arg Phe Ala Phe Arg Ile Tyr Asp Met Asp Lys Asp 100
105 110Gly Tyr Ile Ser Asn Gly Glu Leu Phe Gln Val Leu Lys Met Met Val 115 120 125Gly Asn Asn Leu
Lys Asp Thr Gln Leu Gln Gln Ile Val Asp Lys Thr 130 135 140Ile Ile Asn Ala Asp Lys Asp Gly Asp Gly
Arg Ile Ser Phe Glu Glu145 150 155 160Phe Cys Ala Val Val Gly Gly Leu Asp Ile His Lys Lys Met Val Val
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Thr His Asn Thr Val Arg Gly Cys 245 250 255Ser Tyr Phe Tyr Ser Tyr Pro Ala Val Cys Glu Phe Leu Gln His
Asn 260 265 270Asn Leu Leu Ser Ile Leu Arg Ala His Glu Ala Gln Asp Ala Gly Tyr 275 280 285Arg Met
Tyr Arg Lys Ser Gln Thr Thr Gly Phe Pro Ser Leu Ile Thr 290 295 300Ile Phe Ser Ala Pro Asn Tyr Leu Asp
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Asn Cys 325 330 335Ser Pro His Pro Tyr Trp Leu Pro Asn Phe Met Asp Val Phe Thr Trp 340 345 350Ser
Leu Pro Phe Val Gly Glu Lys Val Thr Glu Met Leu Val Asn Val 355 360 365Leu Asn Ile Cys Ser Asp Asp
Glu Leu Gly Ser Glu Glu Asp Gly Phe 370 375 380Asp Gly Ala Thr Ala Ala Ala Arg Leu Val Thr Ala Gly
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Leu Asp Asn 20 25 30Ser Gly Ser Leu Ser Val Glu Glu Phe Met Ser Leu Pro Glu Leu Gln 35 40 45Gln Asn
Pro Leu Val Gln Arg Val Ile Asp Ile Phe Asp Thr Asp Gly 50 55 60Asn Gly Glu Val Asp Phe Lys Glu Phe
Ile Glu Gly Val Ser Gln Phe65 70 75 80Ser Val Lys Gly Asp Lys Glu Gln Lys Leu Arg Phe Ala Phe Arg Ile
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160His Lys Lys Met Val Val Asp Val 16520745PRTArtificial SequenceGDH-CaM-VHH1 20Asp Val Pro
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Asn Lys Pro His Ala Leu 20 25 30Leu Trp Gly Pro Asp Asn Gln Ile Trp Leu Thr Glu Arg Ala Thr Gly 35 40
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Ile Tyr Ile 85 90 95Ser Gly Thr Phe Lys Asn Pro Lys Ser Thr Asp Lys Glu Leu Pro Asn 100 105 110Gln Thr
Ile Ile Arg Arg Tyr Thr Tyr Asn Lys Ser Thr Asp Thr Leu 115 120 125Glu Lys Pro Val Asp Leu Leu Ala Gly
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Thr145 150 155 160Ile Gly Asp Gln Gly Arg Asn Gln Leu Ala Tyr Leu Phe Leu Pro Asn 165 170 175Gln
Ala Gln His Thr Pro Thr Gln Gln Glu Leu Asn Gly Lys Asp Tyr 180 185 190His Thr Tyr Met Gly Lys Val
Leu Arg Leu Asn Leu Asp Gly Ser Ile 195 200 205Pro Lys Asp Asn Pro Ser Phe Asn Gly Val Val Ser His Ile
Tyr Thr 210 215 220Leu Gly His Arg Asn Pro Gln Gly Leu Ala Phe Thr Pro Asn Gly Lys225 230 235
240Leu Leu Gln Ser Glu Gln Gly Pro Asn Ser Asp Asp Glu Ile Asn Leu 245 250 255Ile Val Lys Gly Gly
Asn Tyr Gly Trp Pro Asn Val Ala Gly Tyr Lys 260 265 270Asp Asp Ser Gly Tyr Ala Tyr Ala Asn Tyr Ser
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Glu Met Ile Arg Glu Ala Asp Ile Asp Gly Asp Gly Gln 450 455 460Val Asn Tyr Glu Glu Phe Val Gln Met
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Ile Thr Gly Trp Glu Asn Thr Leu Leu Val Pro Ser Leu Lys 515 520 525Arg Gly Val Ile Phe Arg Ile Lys Leu
Asp Pro Thr Tyr Ser Thr Thr 530 535 540Tyr Asp Asp Ala Val Pro Met Phe Lys Ser Asn Asn Arg Tyr Arg
Asp545 550 555 560Val Ile Ala Ser Pro Asp Gly Asn Val Leu Tyr Val Leu Thr Asp Thr 565 570 575Ala Gly
Asn Val Gln Lys Asp Asp Gly Ser Val Thr Asn Thr Leu Glu 580 585 590Asn Pro Gly Ser Leu Ile Lys Phe
Thr Tyr Lys Ala Lys Gly Ser Gly 595 600 605Ser Gly Gly Gln Val Gln Leu Val Glu Ser Gly Gly Gly Thr Val
Pro 610 615 620Ala Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Asn Thr Leu625 630 635 640Cys
Thr Tyr Asp Met Ser Trp Tyr Arg Arg Ala Pro Gly Lys Gly Arg 645 650 655Asp Phe Val Ser Gly Ile Asp
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Lys Asn Thr 675 680 685Ala Tyr Leu Gln Met Asp Ser Leu Lys Pro Asp Asp Thr Ala Met Tyr 690 695
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74521116PRTArtificial SequenceVHH2-CaM-BP 21Asp Gly His His His His His His Gly Ser Gly Asp Thr
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Trp Gly 100 105 110Gln Gly Thr Gln Val Thr Val Ser Ser 115 1202374PRTArtificial SequenceVHH2 23Asp
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Gly Cys Ala Glu Thr Val Thr 20 25 30Val Lys Val Val Tyr Glu Asp Asp Thr Glu Gly Leu Cys Tyr Ala Val 35
40 45Ala Pro Gly Gln Ile Thr Thr Val Gly Asp Gly Tyr Ile Gly Ser His 50 55 60Gly His Ala Arg Tyr Leu
Ala Arg Cys Leu65 7024660PRTArtificial SequenceGDH-ePDZ peptide sensor first version 24Asp Val Pro
Leu Ile Pro Ser Gln Phe Ala Lys Ala Lys Ser Glu Asn1 5 10 15Phe Asp Lys Lys Val Ile Leu Ser Asn Leu
Asn Lys Pro His Ala Leu 20 25 30Leu Trp Gly Pro Asp Asn Gln Ile Trp Leu Thr Glu Arg Ala Thr Gly 35 40

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Ile Arg 85 90 95Val Arg Val Glu Lys Asp Gly Gly Ser Gly Gly Val Ser Ser Val Pro 100 105 110Thr Asn Leu
Glu Val Val Ala Ala Thr Pro Thr Ser Leu Leu Ile Ser 115 120 125Trp Asp Ala Tyr Arg Glu Leu Pro Val Ser
Tyr Tyr Arg Ile Thr Tyr 130 135 140Gly Glu Thr Gly Gly Asn Ser Pro Val Gln Glu Phe Thr Val Pro Gly145
150 155 160Ser Lys Ser Thr Ala Thr Ile Ser Gly Leu Lys Pro Gly Val Asp Tyr 165 170 175Thr Ile Thr Val
Tyr Ala His Tyr Asn Tyr His Tyr Tyr Ser Ser Pro 180 185 190Ile Ser Ile Asn Tyr Arg Gly Pro Gly 195
200278PRTArtificial SequencePDZ peptide (ePDZ ligand, high affinity) 27Arg Gly Ser Ile Asp Thr Trp Val1
5288PRTArtificial SequencePDZ peptide (ePDZ ligand, Weak ligand) 28Pro Gln Pro Val Asp Ser Trp Val1
529872PRTArtificial SequenceePDZ-GDH-CalM-Thrombin site-CalM peptide-ePDZ ligand 29Asp His His
His His His His Ser Pro Glu Leu Gly Phe Ser Ile Ser1 5 10 15Gly Gly Val Gly Gly Arg Gly Asn Pro Phe Arg
Pro Asp Asp Asp Gly 20 25 30Ile Phe Val Thr Arg Val Gln Pro Glu Gly Pro Ala Ser Lys Leu Leu 35 40
45Gln Pro Gly Asp Lys Ile Ile Gln Ala Asn Gly Tyr Ser Phe Ile Asn 50 55 60Ile Glu His Gly Gln Ala Val Ser
Leu Leu Lys Thr Phe Gln Asn Thr65 70 75 80Val Glu Leu Ile Ile Val Arg Glu Val Gly Asn Gly Ala Lys Gln
Glu 85 90 95Ile Arg Val Arg Val Glu Lys Asp Gly Gly Ser Gly Gly Val Ser Ser 100 105 110Val Pro Thr Asn
Leu Glu Val Val Ala Ala Thr Pro Thr Ser Leu Leu 115 120 125Ile Ser Trp Asp Ala Tyr Arg Glu Leu Pro Val
Ser Tyr Tyr Arg Ile 130 135 140Thr Tyr Gly Glu Thr Gly Gly Asn Ser Pro Val Gln Glu Phe Thr Val145 150
155 160Pro Gly Ser Lys Ser Thr Ala Thr Ile Ser Gly Leu Lys Pro Gly Val 165 170 175Asp Tyr Thr Ile Thr
Val Tyr Ala His Tyr Asn Tyr His Tyr Tyr Ser 180 185 190Ser Pro Ile Ser Ile Asn Tyr Arg Gly Pro Asp Val
Pro Leu Ile Pro 195 200 205Ser Gln Phe Ala Lys Ala Lys Ser Glu Asn Phe Asp Lys Lys Val Ile 210 215
220Leu Ser Asn Leu Asn Lys Pro His Ala Leu Leu Trp Gly Pro Asp Asn225 230 235 240Gln Ile Trp Leu
Thr Glu Arg Ala Thr Gly Lys Ile Leu Arg Val Asn 245 250 255Pro Glu Ser Gly Ser Val Lys Thr Val Phe Gln
Val Pro Glu Ile Val 260 265 270Asn Asp Ala Asp Gly Gln Asn Gly Leu Leu Gly Phe Ala Phe His Pro 275
280 285Asp Phe Lys Asn Asn Pro Tyr Ile Tyr Ile Ser Gly Thr Phe Lys Asn 290 295 300Pro Lys Ser Thr Asp
Lys Glu Leu Pro Asn Gln Thr Ile Ile Arg Arg305 310 315 320Tyr Thr Tyr Asn Lys Ser Thr Asp Thr Leu Glu
Lys Pro Val Asp Leu 325 330 335Leu Ala Gly Leu Pro Ser Ser Lys Asp His Gln Ser Gly Arg Leu Val 340
345 350Ile Gly Pro Asp Gln Lys Ile Tyr Tyr Thr Ile Gly Asp Gln Gly Arg 355 360 365Asn Gln Leu Ala Tyr
Leu Phe Leu Pro Asn Gln Ala Gln His Thr Pro 370 375 380Thr Gln Gln Glu Leu Asn Gly Lys Asp Tyr His
Thr Tyr Met Gly Lys385 390 395 400Val Leu Arg Leu Asn Leu Asp Gly Ser Ile Pro Lys Asp Asn Pro Ser
405 410 415Phe Asn Gly Val Val Ser His Ile Tyr Thr Leu Gly His Arg Asn Pro 420 425 430Gln Gly Leu Ala
Phe Thr Pro Asn Gly Lys Leu Leu Gln Ser Glu Gln 435 440 445Gly Pro Asn Ser Asp Asp Glu Ile Asn Leu
Ile Val Lys Gly Gly Asn 450 455 460Tyr Gly Trp Pro Asn Val Ala Gly Tyr Lys Asp Asp Ser Gly Tyr Ala465
470 475 480Tyr Ala Asn Tyr Ser Ala Ala Ala Asn Lys Thr Ile Lys Asp Leu Ala 485 490 495Gln Asn Gly Val
Lys Val Ala Ala Gly Val Pro Val Thr Lys Glu Ser 500 505 510Glu Trp Thr Gly Lys Asn Phe Val Pro Pro Leu
Lys Thr Leu Tyr Thr 515 520 525Val Gln Asp Gly Ser Gly Gly Thr Glu Glu Gln Ile Ala Glu Phe Lys 530
535 540Glu Ala Phe Ser Leu Phe Asp Lys Asp Gly Asp Gly Thr Ile Thr Thr545 550 555 560Lys Glu Leu
Gly Thr Val Met Arg Ser Leu Gly Gln Asn Pro Thr Glu 565 570 575Ala Glu Leu Gln Asp Met Ile Asn Glu
Val Asp Ala Asp Gly Asn Gly 580 585 590Thr Ile Asp Phe Pro Glu Phe Leu Thr Met Met Ala Arg Lys Met
Lys 595 600 605Asp Thr Asp Ser Glu Glu Glu Ile Arg Glu Ala Phe Arg Val Phe Asp 610 615 620Lys Asp
Gly Asn Gly Tyr Ile Ser Ala Ala Glu Leu Arg His Val Met625 630 635 640Thr Asn Leu Gly Glu Lys Leu
Thr Asp Glu Glu Val Asp Glu Met Ile 645 650 655Arg Glu Ala Asp Ile Asp Gly Asp Gly Gln Val Asn Tyr
Glu Glu Phe 660 665 670Val Gln Met Met Thr Ala Gly Gly Ser Gly Gly Tyr Asn Tyr Asn Asp 675 680
685Pro Thr Cys Gly Glu Met Thr Tyr Ile Cys Trp Pro Thr Val Ala Pro 690 695 700Ser Ser Ala Tyr Val Tyr
Lys Gly Gly Lys Lys Ala Ile Thr Gly Trp705 710 715 720Glu Asn Thr Leu Leu Val Pro Ser Leu Lys Arg Gly
Val Ile Phe Arg 725 730 735Ile Lys Leu Asp Pro Thr Tyr Ser Thr Thr Tyr Asp Asp Ala Val Pro 740 745
750Met Phe Lys Ser Asn Asn Arg Tyr Arg Asp Val Ile Ala Ser Pro Asp 755 760 765Gly Asn Val Leu Tyr Val
Leu Thr Asp Thr Ala Gly Asn Val Gln Lys 770 775 780Asp Asp Gly Ser Val Thr Asn Thr Leu Glu Asn Pro
Gly Ser Leu Ile785 790 795 800Lys Phe Thr Tyr Lys Ala Lys Leu Val Pro Arg Gly Val Lys Arg Arg 805 810
815Trp Lys Lys Asn Phe Ile Ala Val Ser Ala Ala Asn Arg Phe Lys Lys 820 825 830Ile Ser Gly Gly Ser Gly
Ser Gly Ser Gly Gly Ser Gly Thr Gly Ser 835 840 845Gly Ser Gly Ser Gly Gly Ser Thr Gly Gly Ser Gly Ser
Gly Gly Ser 850 855 860Arg Gly Ser Ile Asp Thr Trp Val865 87030329PRTArtificial SequencePQQ-GDH
residues 1-329 30Asp Val Pro Leu Ile Pro Ser Gln Phe Ala Lys Ala Lys Ser Glu Asn1 5 10 15Phe Asp Lys

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60Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile Ser Val Lys Ser65 70 75 80Glu Lys Ile Ser Thr Leu Ser Ser Glu Asn Lys Ile Ile Ser Phe Lys 85 90 95Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys Ser Asp Ile Ile 100 105 110Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys Met Gln Phe Glu 115 120 125Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Ser Glu Lys Glu Arg Asp 130 135 140Leu Phe Lys Leu Ile Leu Lys Lys Glu Asp Glu Leu Gly Asp Arg Ser145 150 155 160Ile Met Phe Thr Val Gln Asn Glu Asp Gly Ser Gly Ser Gly Ser Gly 165 170 175Ser Gly Ser Gly Gly Arg Gly Ser Ile Asp Thr Trp Val 180 18539690PRTArtificial SequenceAuto-inhibited GDH-ePDZ peptide sensor-strong peptide 39Asp Val Pro Leu Ile Pro Ser Gln Phe Ala Lys Ala Lys Ser Glu Asn1 5 10 15Phe Asp Lys Lys Val Ile Leu Ser Asn Leu Asn Lys Pro His Ala Leu 20 25 30Leu Trp Gly Pro Asp Asn Gln Ile Trp Leu Thr Glu Arg Ala Thr Gly 35 40 45Lys Ile Leu Arg Val Asn Pro Glu Ser Gly Ser Val Lys Thr Val Phe 50 55 60Gln Val Pro Glu Ile Val Asn Asp Ala Asp Gly Gln Asn Gly Leu Leu65 70 75 80Gly Phe Ala Phe His Pro Asp Phe Lys Asn Asn Pro Tyr Ile Tyr Ile 85 90 95Ser Gly Thr Phe Lys Asn Pro Lys Ser Thr Asp Lys Glu Leu Pro Asn 100 105 110Gln Thr Ile Ile Arg Arg Tyr Thr Tyr Asn Lys Ser Thr Asp Thr Leu 115 120 125Glu Lys Pro Val Asp Leu Leu Ala Gly Leu Pro Ser Ser Lys Asp His 130 135 140Gln Ser Gly Arg Leu Val Ile Gly Pro Asp Gln Lys Ile Tyr Tyr Thr145 150 155 160Ile Gly Asp Gln Gly Arg Asn Gln Leu Ala Tyr Leu Phe Leu Pro Asn 165 170 175Gln Ala Gln His Thr Pro Thr Gln Gln Glu Leu Asn Gly Lys Asp Tyr 180 185 190His Thr Tyr Met Gly Lys Val Leu Arg Leu Asn Leu Asp Gly Ser Ile 195 200 205Pro Lys Asp Asn Pro Ser Phe Asn Gly Val Val Ser His Ile Tyr Thr 210 215 220Leu Gly His Arg Asn Pro Gln Gly Leu Ala Phe Thr Pro Asn Gly Lys225 230 235 240Leu Leu Gln Ser Glu Gln Gly Pro Asn Ser Asp Asp Glu Ile Asn Leu 245 250 255Ile Val Lys Gly Gly Asn Tyr Gly Trp Pro Asn Val Ala Gly Tyr Lys 260 265 270Asp Asp Ser Gly Tyr Ala Tyr Ala Asn Tyr Ser Ala Ala Ala Asn Lys 275 280 285Thr Ile Lys Asp Leu Ala Gln Asn Gly Val Lys Val Ala Ala Gly Val 290 295 300Pro Val Thr Lys Glu Ser Glu Trp Thr Gly Lys Asn Phe Val Pro Pro305 310 315 320Leu Lys Thr Leu Tyr Thr Val Gln Asp Glu Asp Ala Pro Glu Ser Gly 325 330 335Ser Pro Glu Leu Gly Phe Ser Ile Ser Gly Gly Val Gly Gly Arg Gly 340 345 350Asn Pro Phe Arg Pro Asp Asp Asp Gly Ile Phe Val Thr Arg Val Gln 355 360 365Pro Glu Gly Pro Ala Ser Lys Leu Leu Gln Pro Gly Asp Lys Ile Ile 370 375 380Gln Ala Asn Gly Tyr Ser Phe Ile Asn Ile Glu His Gly Gln Ala Val385 390 395 400Ser Leu Leu Lys Thr Phe Gln Asn Thr Val Glu Leu Ile Ile Val Arg 405 410 415Glu Val Gly Asn Gly Ala Lys Gln Glu Ile Arg Val Arg Val Glu Lys 420 425 430Asp Gly Gly Ser Gly Gly Val Ser Ser Val Pro Thr Asn Leu Glu Val 435 440 445Val Ala Ala Thr Pro Thr Ser Leu Leu Ile Ser Trp Asp Ala Tyr Arg 450 455 460Glu Leu Pro Val Ser Tyr Tyr Arg Ile Thr Tyr Gly Glu Thr Gly Gly465 470 475 480Asn Ser Pro Val Gln Glu Phe Thr Val Pro Gly Ser Lys Ser Thr Ala 485 490 495Thr Ile Ser Gly Leu Lys Pro Gly Val Asp Tyr Thr Ile Thr Val Tyr 500 505 510Ala His Tyr Asn Tyr His Tyr Tyr Ser Ser Pro Ile Ser Ile Asn Tyr 515 520 525Arg Gly Ser Gly Pro Gly Tyr Asn Tyr Asn Asp Pro Thr Cys Gly Glu 530 535 540Met Thr Tyr Ile Cys Trp Pro Thr Val Ala Pro Ser Ser Ala Tyr Val545 550 555 560Tyr Lys Gly Gly Lys Lys Ala Ile Thr Gly Trp Glu Asn Thr Leu Leu 565 570 575Val Pro Ser Leu Lys Arg Gly Val Ile Phe Arg Ile Lys Leu Asp Pro 580 585 590Thr Tyr Ser Thr Thr Tyr Asp Asp Ala Val Pro Met Phe Lys Ser Asn 595 600 605Asn Arg Tyr Arg Asp Val Ile Ala Ser Pro Asp Gly Asn Val Leu Tyr 610 615 620Val Leu Thr Asp Thr Ala Gly Asn Val Gln Lys Asp Asp Gly Ser Val625 630 635 640Thr Asn Thr Leu Glu Asn Pro Gly Ser Leu Ile Lys Phe Thr Tyr Lys 645 650 655Ala Lys Gly Ser Gly His His His His His His Gly Ser Gly Glu Thr 660 665 670Val Arg Phe Gln Ser Ser Gly Ser Gly Gly Arg Gly Ser Ile Asp Thr 675 680 685Trp Val 69040690PRTArtificial SequenceAuto-inhibited GDH-ePDZ peptide sensor-weak peptide 40Asp Val Pro Leu Ile Pro Ser Gln Phe Ala Lys Ala Lys Ser Glu Asn1 5 10 15Phe Asp Lys Lys Val Ile Leu Ser Asn Leu Asn Lys Pro His Ala Leu 20 25 30Leu Trp Gly Pro Asp Asn Gln Ile Trp Leu Thr Glu Arg Ala Thr Gly 35 40 45Lys Ile Leu Arg Val Asn Pro Glu Ser Gly Ser Val Lys Thr Val Phe 50 55 60Gln Val Pro Glu Ile Val Asn Asp Ala Asp Gly Gln Asn Gly Leu Leu65 70 75 80Gly Phe Ala Phe His Pro Asp Phe Lys Asn Asn Pro Tyr Ile Tyr Ile 85 90 95Ser Gly Thr Phe Lys Asn Pro Lys Ser Thr Asp Lys Glu Leu Pro Asn 100 105 110Gln Thr Ile Ile Arg Arg Tyr Thr Tyr Asn Lys Ser Thr Asp Thr Leu 115 120 125Glu Lys Pro Val Asp Leu Leu Ala Gly Leu Pro Ser Ser Lys Asp His 130 135 140Gln Ser Gly Arg Leu Val Ile Gly Pro Asp Gln Lys Ile Tyr Tyr Thr145 150 155 160Ile Gly Asp Gln Gly Arg Asn Gln Leu Ala Tyr Leu Phe Leu Pro Asn 165 170 175Gln Ala Gln His Thr Pro Thr Gln Gln Glu Leu Asn Gly Lys Asp Tyr 180 185 190His Thr Tyr Met Gly Lys Val Leu Arg Leu Asn Leu Asp Gly

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Val Glu 165 170 175Thr Ile Ser Pro Gly Asp Gly Arg Thr Phe Pro Lys Arg Gly Gln Thr 180 185 190Cys Val
Val His Tyr Thr Gly Met Leu Glu Asp Gly Lys Lys Phe Asp 195 200 205Ser Ser Arg Asp Arg Asn Lys Pro
Phe Lys Phe Met Leu Gly Lys Gln 210 215 220Glu Val Ile Arg Gly Trp Glu Glu Gly Val Ala Gln Met Ser
Val Gly225 230 235 240Gln Arg Ala Lys Leu Thr Ile Ser Pro Asp Tyr Ala Tyr Gly Ala Thr 245 250 255Gly
His Pro Gly Ile Ile Pro Pro His Ala Thr Leu Val Phe Asp Val 260 265 270Glu Leu Leu Lys Leu Glu Gly Ser
Gly Gln Lys Ile Tyr Tyr Thr Ile 275 280 285Gly Asp Gln Gly Arg Asn Gln Leu Ala Tyr Leu Phe Leu Pro
Asn Gln 290 295 300Ala Gln His Thr Pro Thr Gln Gln Glu Leu Asn Gly Lys Asp Tyr His305 310 315
320Thr Tyr Met Gly Lys Val Leu Arg Leu Asn Leu Asp Gly Ser Ile Pro 325 330 335Lys Asp Asn Pro Ser
Phe Asn Gly Val Val Ser His Ile Tyr Thr Leu 340 345 350Gly His Arg Asn Pro Gln Gly Leu Ala Phe Thr Pro
Asn Gly Lys Leu 355 360 365Leu Gln Ser Glu Gln Gly Pro Asn Ser Asp Asp Glu Ile Asn Leu Ile 370

375 380Val Lys Gly Gly Asn Tyr Gly Trp Pro Asn Val Ala Gly Tyr Lys Asp385 390 395 400Asp Ser Gly Tyr
Ala Tyr Ala Asn Tyr Ser Ala Ala Ala Asn Lys Thr 405 410 415Ile Lys Asp Leu Ala Gln Asn Gly Val Lys Val
Ala Ala Gly Val Pro 420 425 430Val Thr Lys Glu Ser Glu Trp Thr Gly Lys Asn Phe Val Pro Pro Leu 435 440
445Lys Thr Leu Tyr Thr Val Gln Asp Thr Tyr Asn Tyr Asn Asp Pro Thr 450 455 460Cys Gly Glu Met Thr
Tyr Ile Cys Trp Pro Thr Val Ala Pro Ser Ser465 470 475 480Ala Tyr Val Tyr Lys Gly Gly Lys Lys Ala Ile
Thr Gly Trp Glu Asn 485 490 495Thr Leu Leu Val Pro Ser Leu Lys Arg Gly Val Ile Phe Arg Ile Lys 500 505
510Leu Asp Pro Thr Tyr Ser Thr Thr Tyr Asp Asp Ala Val Pro Met Phe 515 520 525Lys Ser Asn Asn Arg
Tyr Arg Asp Val Ile Ala Ser Pro Asp Gly Asn 530 535 540Val Leu Tyr Val Leu Thr Asp Thr Ala Gly Asn
Val Gln Lys Asp Asp545 550 555 560Gly Ser Val Thr Asn Thr Leu Glu Asn Pro Gly Ser Leu Ile Lys Phe
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