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Supporting Information

Heterogeneous Reconstitution of the PQQ-Dependent Glucose Dehydrogenase Immobilized on an Electrode: A Sensitive Strategy for PQQ Detection Down to Picomolar Levels

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Biotinylation of apo-GDH. Two different *N*-hydroxysuccinimide esters of biotin (i.e., the sulfosuccinimidyl-6-[biotin-amido]hexanoate and the NHS-PEG_{12}-biotin) were used as biotinylation reagents. Both react with primary amino groups on the protein surface to form stable amide bonds. For the coupling reaction, an amine-free buffer is required to avoid competition with the intended reaction. For that reason, the mother solution of apo-GDH was first exchanged with PBS by centrifugation (11 000 × *g*, 30 min) on a Nanosep membrane of 10 kDa MWCO (Pall Corporation). To determine the optimal conditions of enzyme biotinylation, various molar excesses of biotinylation reagent with respect to the dimer enzyme concentration (40 µM dimer in PBS) were tested (i.e., molar excess of 40, 12, and 5). The optimal condition was finally found for an excess ratio of 5 (see Results and Discussions). After 2 hrs incubation at 4°C, the biotinylated protein was next separated from the reaction mixture by centrifugation on a Nanosep membrane, washed twice with TB and once with EB, and finally redispersed in EB. In this latter buffer, the b-apo-GDH is known to stabilize in the dimeric form.[S1] The concentration of biotinylated apo-GDH (dimer) was determined spectrophotometrically by considering a molar extinction coefficient of 128 000 M^{-1} cm^{-1} at λ = 280 nm.[S1, 46] The resulting activity of the b-apo-GDH was determined after its reconstitution with PQQ as described below.

Homogeneous reconstitution of PQQ-GDH from b-apo-GDH. The protocol for homogeneous reconstitution of biotinylated PQQ-GDH (b-holo-GDH) from the corresponding biotinylated apoenzyme form was carried out according to a slightly modified published protocol.[22, 23] Briefly, the b-apo-GDH (40 µM monomer) was mixed with a 20-fold excess of PQQ (calculated relative to the subunit concentration of apo-GH) in EB and incubated for 30 min at room temperature (the concentration of PQQ was determined spectrophotometrically using a molar extinction coefficient of 19 000 M^{-1} cm^{-1} at 250 nm). Then, excess of PQQ was removed by centrifugation (11 000 × *g*, 30 min) using a Nanosep membrane of 10 kDa MWCO (Pall Corporation) pre-equilibrated with EB. The reconstituted PQQ-GDH was next

S1. Olsthoorn A. J. J.; Otsuki, T.; Duine, J. A. *Eur. J. Biochem.,* **1997**, *247,* 659-65.

washed three times with EB and finally redispersed in the same buffer. Incorporation of PQQ within the protein scaffold was confirmed from the UV-vis spectrum, showing a peak at λ = 352 nm (Figure S1). Subsequent addition of 1 mM glucose led to a slight increase and blue shift in the absorption maximum (i.e., λ = 338 nm), characteristic of the absorbance of the reduced form of bound PQQ (Figure S1). The ratio between the absorbance at 338 and 280 nm (A_{338}/A_{280}) was taken as an indicator of the relative amount of active enzyme. Values ranging from 0.47 to 0.50 were found, in agreement with that reported in ref 23. The resulting concentration of glucose-reduced holo-GDH (dimer) was determined using a molar extinction coefficient of 174 000 M^{-1} cm^{-1} at λ = 280 nm. Once prepared the reconstituted enzyme was aliquoted and stored at -20 °C. The activity of the reconstituted b-holo-GDH was determined as previously[22] by monitoring in CV (0.1 V/s) the steady-state catalytic current response of a diluted solution of b-holo-GDH (0.1 µM in TB-BSA) containing various concentrations of FcMeOH and glucose.

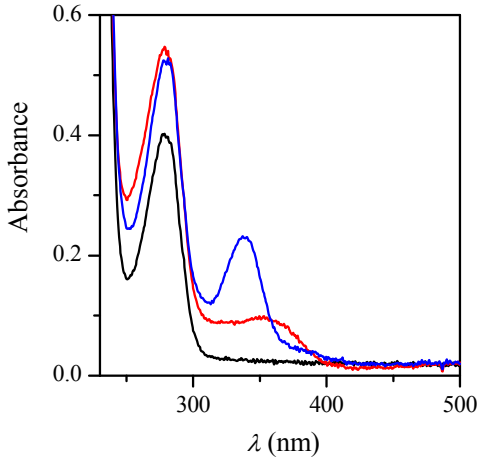

Figure S1. UV-visible spectra of (black curve) b-apo-GDH, (red curve) b-holo-GDH, and (blue curve) glucose reduced b-holo-GDH (i.e., after addition of 1 mM D-glucose to the cuvette).

Determination of the maximal surface concentration of b-holo-GDH. The maximal amount of b-holo-GDH that can be specifically bounded to a neutravidin-modified GC electrode was independently determined using the *droplet depletion method* previously developed by us for horseradish peroxidase[44] and extended later for alkaline phosphatase.[39] Basically, the method consists in depositing a droplet of

biotinylated enzyme solution on the surface of a neutravidin-modified electrode and, after an incubation step, in determining spectrophotometrically the enzyme activity remaining in the droplet solution. On account of the large electrode surface/droplet volume ratio, a sizable amount of biotinylated enzyme is depleted from the drop. This amount normalized with respect to the geometric electrode area would therefore correspond to the enzyme specifically attached to the electrode surface, i.e. Γ^0_{holo}. The details of the procedure are as follow. After the 5-μL droplet containing the unbounded biotinylated PQQ-GDH was collected in a 0.5 mL EB-BSA, the enzyme activity of this diluted solution was analyzed spectrophotometrically by monitoring the absorbance decrease of the 2,6-dichlorophenolindophenol (DCPIP) in the presence of glucose and phenazine methosulfate (PMS) as primary electron acceptor.[20] This was achieved by further diluting the solution of unbounded b-holo-GDH in assay solutions (2 mL) containing 0.2 mM PMS, 0.1 mM DCPIP and 5 mM glucose in EB-BSA, followed by measurement of the absorbance decreases at λ = 600 nm for a few minutes. From comparison of the initial rate of absorbance change with those obtained for standard diluted solutions of b-holo-GDH under same conditions, it was then possible to determine the concentration of unbounded b-holo-GDH in the collected 5-μL droplet and by difference to inferred the amount of b-holo-GDH that was specifically bound to the electrode surface. To consider possible loss of enzyme during the course of immobilization (e.g., by denaturation of the enzyme at the liquid/air or solid/liquid interfaces), control experiments were carried out with droplets of biotinylated enzyme solution incubated with BSA-modified GC electrodes. After correction from enzyme loss, an average biotinylated PQQ surface concentration of Γ^0_{holo} = 1.1 ± 0.1 pmol cm^{-2} was finally found.

Determination of the homogeneous affinity binding constant of PQQ for the apo-GDH binding sites. Solutions of apo-GDH (final concentrations of 125 pM of empty PQQ binding sites) were mixed with freshly made PQQ solutions (final concentrations ranging from 3 × 10^{-11} M to 2.5 × 10^{-9} M) in disposable cuvettes (PMMA cuvettes for UV-visible spectroscopy, optical pathlength: 10 mm) containing EB and 0.1 wt% Triton X-100 (total reaction volume of 1.6 mL), and then left to react for 1 hr at room

temperature. Disposable cuvettes based on poly(methylmethacrylate) were systematically used for these experiments in order to avoid the non-negligible adsorption of PQQ on glass vials or quartz cuvettes. After incubation, the reaction volume was completed to 2 mL by the addition of 50 µM DCPIP, 1 mM PMS and 5 mM glucose, and the fraction of reconstituted holo-GDH then spectrophotometrically determined from the discoloration rate of DCPIP at 600 nm. The resulting initial rates of absorbance decrease were normalized to the one determined for a standard solution of 50 pM native PQQ-GDH (dimer) and plotted as a function of PQQ concentration. The normalized plot (Figure S2) was fitted with a simple mass action law and the affinity binding constant (K_b) of PQQ for the apo-GDH binding sites inferred.

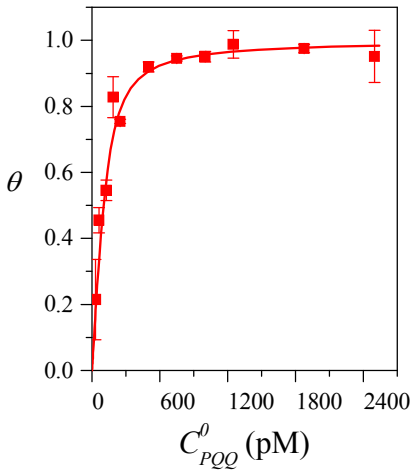

Figure S2. Normalized enzyme activity of PQQ-GDH after homogeneous reconstitution with different concentrations of PQQ. The starting concentration of apo-GDH in each experiment was 125 pM of binding sites. Errors bars represent the standard deviation over 2–4 experimental data. Red line represents the theoretical fit based on mass action law and a binding constant of $K_b = (3 \pm 1.5) \times 10^{10}$ M^{-1}.

Transient and steady-state kinetics of the homogenous reconstitution of PQQ-GDH. The transient formation rate of the reduced form of PQQ-GDH was determined by single turnover stopped-flow experiments at 338 nm. Measurements were carried out at 10°C on a MOS-450 stopped-flow apparatus (Biologic) and the collected data were analyzed using the Bio-kine 32 V4.70 software package. Equal volumes (63 µL) of a solution containing 5 µM apo-GDH (in 50 mM Tris buffer, pH 7.5) and a solution

containing 0.2 mM glucose with various concentrations of PQQ ranging from 5 to 80 μM (in 50 mM Tris, 6 mM $CaCl_2$, pH 7.5) were mixed. Rate constants, k_{obs}, were obtained by fitting the absorbance at 338 nm with an exponential rate law. Each data has been reproduced four times and for two different batches of enzyme. To ensure that the process followed at 338 nm was exclusively controlled by the reconstitution reaction and not by the enzymatic glucose oxidation, a control experiment was carried out with the native enzyme under the same conditions but without PQQ. The time response for complete reduction of the native PQQ-GDH was finally found much shorter than the time responses obtained with the single-turnover enzyme reconstitution experiments. The bimolecular rate constant, k_{on}, resulting from the reconstitution/activation of the enzyme was then recovered from the linear regression fit of the plot of k_{obs} as a function of PQQ concentration.

The homogeneous reconstitution rate of PQQ-GDH was also determined from steady-state kinetics experiments. This was achieved from the spectrophotometric monitoring of the time-course of DCIP discoloration during the reconstitution reaction of apo-GDH with PQQ (at catalytic concentrations of enzyme compared to the substrates in excess). For such a purpose, in disposable PMMA cuvettes 200 μL of a diluted PQQ solution (final concentrations ranging from 2×10^{-10} M to 1×10^{-8} M) was rapidly injected and mixed to a 1.8 mL solution containing 50 pM apo-GDH, 50 μM DCPIP, 1 mM PMS, 5 mM glucose (in EB + 0.1 % Triton X-100). Immediately after mixing, the discoloration was followed as a function of time at λ = 600 nm. To extract the kinetics information contained in the nonlinear time-course change of absorbance, the experimental kinetics plots were fitted to the simulated ones (Figure S3). For the simulations, we have used the GEPASI 3.30 freeware,[S2] a digital simulation program for homogenous biochemical reactions. The reaction scheme input into the GEPASI software to simulate and fit the experimental plots in Figure S3 was based on the following coupled PQQ binding and enzyme catalytic reaction scheme:

S2. Mendes, P., *Trends Biochem. Sci.,* **1997**, *22*, 361–3.

(i) PQQ binding/enzyme reactivation process:

$$E_i + PQQ \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} E_a$$

(where E_i and E_a are the inactive and activated enzyme binding sites, respectively)

(ii) catalytic enzyme reaction based on a simple apparent Michaelis-Menten mechanism:

$$E_a + S \underset{k_{-j}}{\overset{k_j}{\rightleftarrows}} E_aS$$

$$E_aS \xrightarrow{k_{cat}} E_a + P$$

where S is the colored oxidized form of DCIP that is enzymatically converted into uncolored P. This minimal enzyme mechanism is ample to account for the steady-state catalytic reaction of PQQ-GDH at a given set of glucose (in excess), PMS and DCIP concentrations.

(iii) first-order nonspecific discoloration of the solution (recorded in the absence of apo-GDH):

$$S \xrightarrow{k_{disp}} P$$

(The following set of constants was used to generate the simulated plots in Figure S3: $k_{on} = 8 \times 10^5$ $M^{-1}s^{-1}$, $k_{off} = 2.6 \times 10^{-5}$ s^{-1}, $k_j = 10^9$ $M^{-1}s^{-1}$, $k_{-j} = 1$ s^{-1}, $k_{cat} = 3100$ s^{-1}, $k_{disp} = 10^{-4}$ s^{-1}).

From the best fits, a homogenous binding rate constant of $k_{on} = 8 \times 10^5$ $M^{-1}s^{-1}$ was finally obtained.

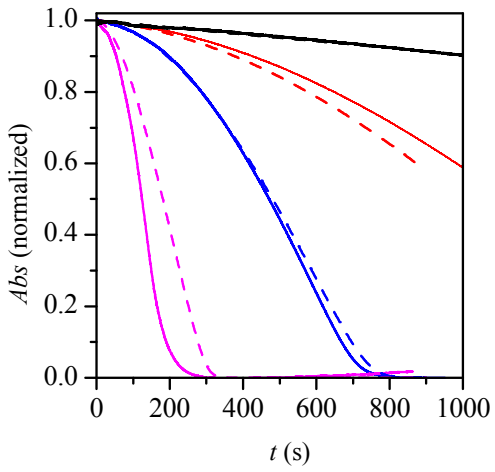

Figure S3. Normalized absorbance change at $\lambda = 600$ nm as a function of time after the addition of different concentrations of PQQ: (black) 0 nM, (red) 0.2 nM, (blue) 1 nM, and (pink) 10 nM. The starting concentration of apo-GDH was 0.1 nM binding sites. Nonspecific discoloration of DCIP in presence of PMS was evaluated from the plot at 0 nM PQQ. Specific discoloration by

the activated enzyme was estimated when holo-GDH was used instead of apo-GDH under the same experimental conditions. Dashed lines represent the simulated curves generated with the GEPASI simulation software.

Establishment of equation 5. During a cyclic voltammetric experiment, the mediator R (FcMeOH) introduced in solution at a concentration C_R^0, diffuses toward the electrode surface where it is oxidized to give O (Fc^+MeOH):

$$R - e^- \rightleftharpoons O$$

O can either diffuse back to the solution or react in the vicinity of the electrode surface with the substrate through enzyme catalysis leading to the regeneration of R. The rate of regeneration of R in the enzyme film depends only on the enzymatic rate when assuming that glucose, the enzyme substrate, is not significantly depleted within the enzyme reaction layer (in that case the substrate diffusion is not the limiting rate and we can consider that its concentration at the electrode surface is always equal to its concentration in the bulk, so that at $x = 0$, $[G]_{x=0} = C_G^0$). When mutual compensation between oxidation and catalytic regeneration of R occurs, this leads to a steady-state situation. In that case, the catalytic current will exhibit a plateau rather than a peak shape.

First, the rate of the enzymatic reaction should be expressed as a function of known constants and parameters. Under the conditions of non-cooperative mode (i.e., low ferrocene methanol concentration), the enzymatic rate can be derived from the following set of surface reactions (for simplicity, we have redefined the different enzyme forms involved in reactions 1-4 of the manuscript by E_1, E_2, E_3 and E_4):

$$G + E_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_2$$

$$E_2 \xrightarrow{k_c} Glu + E_3$$

$$2\,O + E_3 \xrightarrow{k_{ox}} 2\,R + E_1$$

$$E_3 + G \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} E_4$$

Application of the steady-state approximation to all enzyme forms leads to the following set of four equations:

$$\frac{d\Gamma_{E_1}}{dt} = k_{ox}[O]_{x=0}\Gamma_{E_3} - k_1 C_G^0 \Gamma_{E_1} + k_{-1}\Gamma_{E_2} = 0$$
$$\frac{d\Gamma_{E_2}}{dt} = k_1 C_G^0 \Gamma_{E_1} - k_{-1}\Gamma_{E_2} - k_c \Gamma_{E_2} = 0$$
$$\frac{d\Gamma_{E_3}}{dt} = k_c \Gamma_{E_2} - k_{ox}[O]_{x=0}\Gamma_{E_3} - k_i C_G^0 \Gamma_{E_3} + k_{-i}\Gamma_{E_4} = 0$$
$$\frac{d\Gamma_{E_4}}{dt} = k_i \Gamma_{E_3} - k_{-i}\Gamma_{E_4} = 0$$

of which only three are independent. Application of the steady-state approximation to the substrate inhibited form of the enzyme is justified by the fact that the catalytic activity does not decrease during the cyclic voltammetric experiments and leads to the characterization of inhibition through equilibrium constants rather than rate constants:

$$\Gamma_{E_4} = \frac{k_i C_G^0 \Gamma_{E_3}}{k_{-i}} = K_i C_G^0 \Gamma_{E_3}$$

Conservation of the total enzyme concentration in solution leads to: $\Gamma_{E_1} + \Gamma_{E_2} + \Gamma_{E_3} + \Gamma_{E_4} = \Gamma_{holo}^0$.

At the electrode surface, the enzyme rate can be expressed as the rate of one of the three reactions constituting the main catalytic cycle:

$$rate = k_1 C_G^0 \Gamma_{E_1} = k_c \Gamma_{E_2} = k_{ox}[O]_{x=0}\Gamma_{E_3}$$

Besides, by combining and substituting linearly all above equations, we can express the enzyme rate as a function of the total enzyme concentration, as well as a function of the different kinetic rate and equilibrium constants, and substrate and mediator concentrations:

$$rate = \frac{\Gamma_{holo}^0}{\frac{1}{k_c} + \frac{K_M}{k_c C_G^0} + \frac{1}{k_{ox}[O]_{x=0}} + \frac{K_i C_G^0}{k_{ox}[O]_{x=0}}} \quad \text{(S1)}$$

As mentioned above, the R consumption/O regeneration balance within the enzyme layer result in the following expression of the current:[40]

$$\frac{i}{FS} = -D_R\left(\frac{\partial[O]}{\partial x}\right)_{x=0} + 2 \times rate \quad \text{(S2)}$$

where S is the electrode area and D_R the diffusion coefficient of O. This equation links the flux of O produced at the electrode by oxidation of R (left-hand side of the equation) to the flux of O that either diffuse from the electrode towards the solution or is involved in the enzyme catalytic cycle (right-hand side of the equation). The stoichiometric factor 2 stands for the fact that two molecules of O are required to oxidize one substrate molecule, so that two R molecules are regenerated by enzyme catalytic cycles.

Finally, from the combination of eqs S1 and S2, the expression of the current is given by:

$$\frac{i}{FS} = -D_R\left(\frac{\partial[\mathrm{O}]}{\partial x}\right)_{x=0} + \frac{2\,\Gamma^0_{holo}}{\frac{1}{k_c} + \frac{K_M}{k_c C^0_G} + \frac{1}{k_{ox}[\mathrm{O}]_{x=0}} + \frac{K_i C^0_G}{k_{ox}[\mathrm{O}]_{x=0}}} \qquad \text{(S3)}$$

where $[\mathrm{O}]_{x=0}$ is the concentration of Fc^+MeOH electrochemically generated at the electrode surface (i.e., at $x = 0$). Eq S3 is in reality the combination of two terms, the first one related to the diffusion current of the redox mediator and the second one associated to the enzyme catalysis. Because plateau currents are recorded at anodic potentials well beyond the standard potential of mediator (for FcMeOH, $E^0 = 0.16$ V *vs.* ECS), we can assumed that at $x = 0$, $[\mathrm{O}]_{x=0} = C^0_R$ (with C^0_R the bulk concentration of FcMeOH). Subtraction of the diffusive CV response of mediator from the total current therefore leads to eq 5 expressing the catalytic steady-state plateau current density, j_{pl}:

$$j_{pl} = 2\,F\,\frac{\Gamma^0_{holo}}{\frac{1}{k_c} + \frac{K_M}{k_c C^0_G} + \frac{1}{k_{ox} C^0_R} + \frac{K_i C^0_G}{k_{ox} C^0_R}}$$

Table S1. **Kinetics constants of PEG_{12}-biotinylated PQQ-GDH**

		Homogeneous	Heterogeneous
Non cooperative mode	k_{ox} ($M^{-1} s^{-1}$)	$(1.8 \pm 0.5)\times10^{8}$	$(1.4 \pm 0.2)\times10^{8}$
	k_c (s^{-1})	1500 ± 300	1500 ± 400
	K_M (M)	$(3.6 \pm 0.6)\times10^{-4}$	$(4.1 \pm 0.9)\times10^{-4}$
	K_i (M^{-1})	47 ± 3	27 ± 5
Cooperative mode	k''_{ox} ($M^{-1} s^{-1}$)	$(2.7 \pm 0.3)\times10^{8}$	n.d.
	k''_c (s^{-1})	6000 ± 1000	n.d.
	K''_M (M)	$(5 \pm 1)\times10^{-3}$	n.d.
	K''_i (M^{-1})	88 ± 20	n.d.

n.d.: not determined

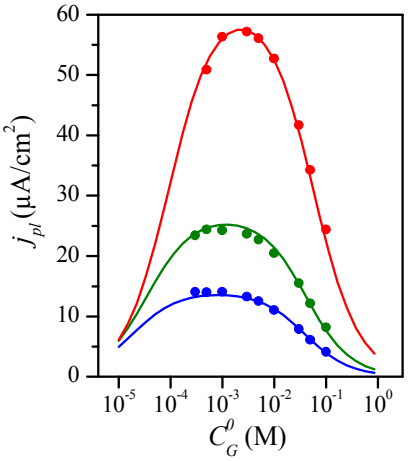

Figure S4. Catalysis of glucose oxidation by immobilized PEG_{12}-biotinylated PQQ-GDH on glassy carbon electrodes in 0.1 M EB (pH 7.5). Steady-state plateau currents as a function of glucose concentration for 0.5 µM (blue), 1 µM (olive) and 3 µM (red) of FcMeOH. Plain lines are theoretical curves corresponding to eq 5 using kinetic constants reported in Table S1 and an enzyme surface coverage of 1.1 pmol/cm².