Electrical Wiring of Glucose Oxidase by Reconstitution of FAD-Modified Monolayers Assembled onto Au-Electrodes

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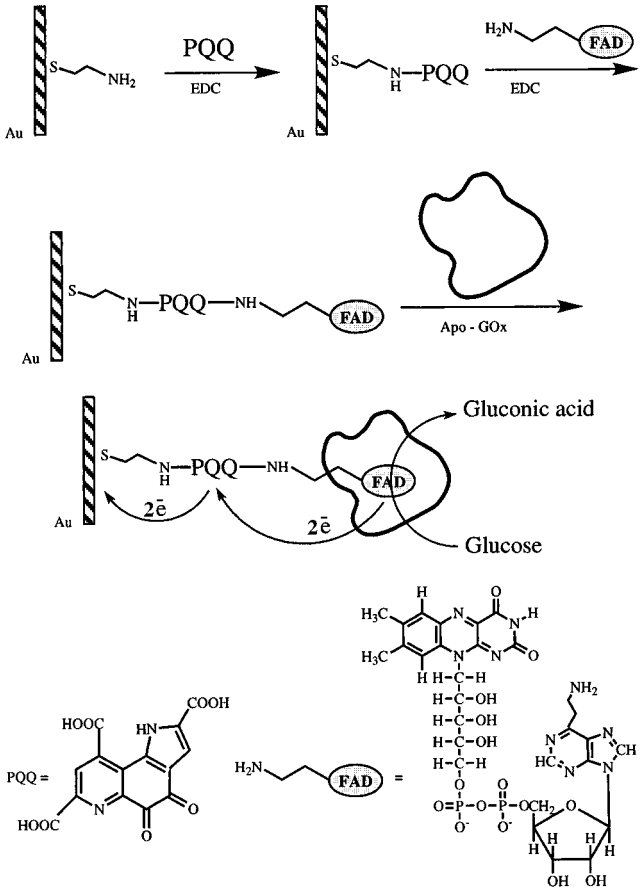
The study of direct electrical communication between reaction centers of enzymes and electrodes bridges fundamental work on electron transfer through proteins and practical work on biosensors.[1,2] Such electrical communication has been realized earlier through modification of enzymes with electron relays,[3,4] through modification of their peripheral oligosaccharides with relays pendant on termini of flexible spacer chains,[5] and through relays in electron-conducting hydrogels within which enzymes were covalently bound.[6] Recently, electrical communication, through a defined molecular path, was achieved by reconstituting apo-glucose and apo-D-amino acid oxidases with a ferrocene-modified FAD cofactor.[7] Here we report a novel method to assemble an enzyme electrode by reconstitution of apo-glucose oxidase on a pyrroloquinoline quinone/FAD (PQQ/FAD) monolayer associated with an Au-electrode. The resulting enzyme layer reveals efficient electrical contact with the electrode and stimulates effectively the electrobiocatalyzed oxidation of glucose.

The monolayer of glucose oxidase (GOx) electrically connected to the Au-electrode, was formed as shown in Scheme 1. A base cystamine submonolayer was bound to a roughened gold foil having a solution-exposed surface area of 6 ± 2 cm^2 (0.4 ± 0.04 cm^2 geometric area, 15 ± 5 roughness coefficient).[8] The base layer was first reacted with pyrroloquinoline quinone (PQQ),[9] and the resulting PQQ submonolayer was then reacted with N^6-(2-aminoethyl)-FAD to yield the PQQ/FAD diad layer.

Scheme 1. Reconstitution of Glucose Oxidase onto a PQQ/FAD Monolayer Au-Electrode and Direct Electrocatalyzed Oxidation of Glucose by the Modified Electrode



The diad exhibited at pH = 7 the two expected redox waves, one characteristic of the PQQ/PQQH$_2$ functions at −0.125 V (vs. SCE) and the second characteristic of FAD/FADH$_2$ at −0.50 V (vs. SCE). Integration of the redox waves obtained by voltammetry showed the presence of 5.5×10^{-10} mol cm^{-2} of both PQQ/PQQH$_2$ and FAD/FADH$_2$, both in excess of the 1.7×10^{-12} mol cm^{-2} of GOx that can be maximally packed in a monolayer. Treatment of the submonolayer with apo-glucose oxidase[10] produced the reconstituted electrically connected and densely packed GOx monolayer. Because part of the FAD/FADH$_2$ functions was now bound within the electrically insulating glycoprotein of the enzyme, the FAD/FADH$_2$ redox waves were suppressed, while the PQQ/PQQH$_2$ waves were not.[11] The surface coverage of the Au-surface by the reconstituted GOx was determined by microgravimetric, quartz crystal microbalance (QCM) analysis. The PQQ/FAD diad was assembled onto Au-electrodes (0.2 cm^2, roughness factor 3) associated with a quartz crystal (9 MHz). From the observed frequency change of the crystal upon reconstitution of apo-GOx on the surface ($\Delta f = -150$ Hz), the surface coverage of the enzyme was calculated to be 1.5×10^{-12} mol cm^{-2}, consistent

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(10) Reconstitution of the enzyme on the electrode was accomplished by shaking the PQQ/FAD monolayer electrode with apo-GOx, 4 mg mL^{-1} in 0.1 M phosphate buffer, pH = 7.0, for 4 h at 25 °C and 12 h at 4 °C. The electrode was rinsed by shaking the resulting electrode for 1 h in 0.1 M phosphate buffer, pH = 7.0, at 0 °C.

(11) Coulometric assay of the FAD/FADH$_2$ and PQQ/PQQH$_2$ waves after reconstitution with apo-GOx reveal that only ca. 10% of the FAD units are electrically accessible and the remainder are electrically insulated. We assume that not only FAD/FADH$_2$ units bound within the protein but also external free FAD sites are electrically shielded by the reconstituted protein.

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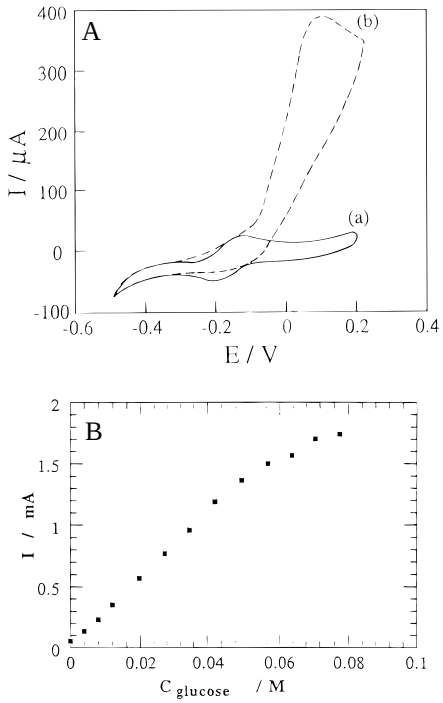

Figure 1. (A) Cyclic voltammograms of (a) glucose oxidase reconstituted with the PQQ/FAD monolayer bound to the gold electrode and (b) after adding glucose to 80 mM concentration. Conditions: Ar atmosphere; 0.01 M phosphate buffer and 0.1 M sodium sulfate, pH = 7.0; 35 °C; scan rate 5 mV s^{-1}; electrode geometrical area, 0.4 cm^2; actual area 6 ± 2 cm^2 (roughness factor 15 ± 5). (B) Glucose concentration dependence of the electrooxidation current for the PQQ-reconstituted GOx monolayer electrode. Currents determined by chronoamperometry at final potential +0.2 V. Conditions: 35 °C; electrode geometrical area 0.4 cm^2; actual area 6 ± 2 cm^2.

with the calculated value for a maximally packed GOx monolayer (*vide infra*).

The reconstituted GOx monolayer is electrically connected with the electrode by the PQQ redox relay, and in the presence of glucose, electrobiocatalyzed oxidation of the substrate takes place as indicated by the electrocatalytic anodic current (Figure 1A), whereas the PQQ/FAD monolayer alone does not stimulate oxidation of glucose. Figure 1B shows the anodic currents of the enzyme electrode at different glucose concentrations. A linear current increase is observed up to a glucose concentration of ca. 80 mM, and then the current levels off. The electrically connected GOx monolayer produced a current density corresponding to the theoretical limit for the maximum possible turnover rate of a densely packed monolayer of glucose oxidase at 35 °C. The current density, before taking into account the 15 ± 5 ratio of the actual/geometrical area of the roughened gold electrode, reached 4.5 mA cm^{-2} (i.e., the actual current density was 300 ± 100 μA cm^{-2}, Figure 1B). The theoretical limit of the current density obtainable for a densely packed glucose oxidase monolayer, 290 ± 60 μA cm^{-2}, was calculated as follows. The enzyme has a footprint of 58 nm^2,[12] which allows at the ordered packing limit 2.9 × 10^{-12} mol cm^{-2} of enzyme. Random, disordered packing allows only 60% coverage (i.e., 1.7 × 10^{-12} mol cm^{-2}).[13,14] The upper limit of the turnover rate at 25 °C is 600 ± 100 s^{-1},[13] and the activation energy is 7.2 kcal mol^{-1}.[15] These values translate at 35 °C, the temperature of our measurements, to a turnover rate of 900 ± 150 s^{-1}. This upper limit of turnover rate occurs when several conditions are met: the glucose concentration is so high that nearly all (>95%) of the FAD (i.e., oxidized) enzyme is glucose complexed (>80 mM in our case); the electrode is poised at a sufficiently positive potential for nearly all relays to be in the oxidized state (>0.2V (vs. SCE) in our case); all reaction centers are intact and connected. The measured current density, 300 ± 100 μA cm^{-2} at 35 °C, shows that all or most of the reaction centers were intact and connected.[16] Control experiments revealed that PQQ is an essential component to establish electrical contact between the FAD centers and the electrode. Direct covalent attachment of N^6-(2-aminoethyl)-FAD to a lipoic acid monolayer assembled onto an Au-electrode does not yield an enzyme monolayer exhibiting electrical contact with the electrode after reconstitution with apo-GOx. Thus, non-reconstituted FAD units in the monolayer array do not mediate electron transfer to FAD sites embedded in the protein.[17]

Consistently with fast rate of electron transfer from the $FADH_2$ centers to the electrode, the glucose electrooxidation current was limited in the physiologically relevant 2−50 mM range by the diffusion of glucose to the electrode surface, not by the kinetics of the electrocatalytic process. Furthermore, oxygen, the natural cosubstrate of GOx, is a characteristic current quencher of a glucose-sensing electrode. The effective electrical contact between the PQQ/FAD-reconstituted GOx and the electrode suggests minor interference of O_2 on the amperometric response. Indeed, the current response of the enzyme electrode at 5 mM glucose was decreased by less than 5% in an aerated solution as compared to that in an argon-flushed solution. Similarly, ascorbate, a particularly rapidly electrooxidized interferant in amperometric glucose assays, was not electrooxidized preferentially over glucose. At 0.1 mM concentration, typical of its physiological level, it did not measurably affect the electrooxidation current of 5 mM glucose.

We conclude that the novel method to assemble a GOx monolayer electrode via reconstitution of the apo-protein with the PQQ/FAD monolayer, yields a functionalized electrode for electrooxidation of glucose at an unprecendentally high rate, reaching the theoretical limit for a maximally packed enzyme monolayer. The fast electrochemical kinetics between the immobilized enzyme and the electrode make the system remarkable both in its transduced current densities and selectivity. These two elements suggest the potential invasive use of a miniaturized reconstituted enzyme electrode for *in vivo* continuous monitoring of glucose levels.[18]

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Supporting Information Available: Cyclic voltammograms of PQQ/FAD monolayer electrode before and after the reconstitution with apo-GOx are presented (1 page). See any current masthead page for ordering and Internet access instructions.

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(16) We assume that reconstitution of apo-GOx on the surface includes the incorporation of two FAD units into the two subunits of the protein.[12] The calculation of the theoretical limiting current density assumes the participation of two active subunits of GOx. From the experimental QCM surface coverage of the reconstituted GOx and observed current density, both of the subunits are activated.

(17) The reconstituted GOx on the FAD−lipoic acid monolayer is assembled in a bioactive protein configuration. The enzyme electrode stimulates the bioelectrocatalyzed oxidation of glucose in the presence of ferrocene carboxylic acid as diffusional electron mediator.

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