Reconstitution of Apo-Glucose Oxidase with a Nitrospiropyran-Modified FAD Cofactor Yields a Photoswitchable Biocatalyst for Amperometric Transduction of Recorded Optical Signals

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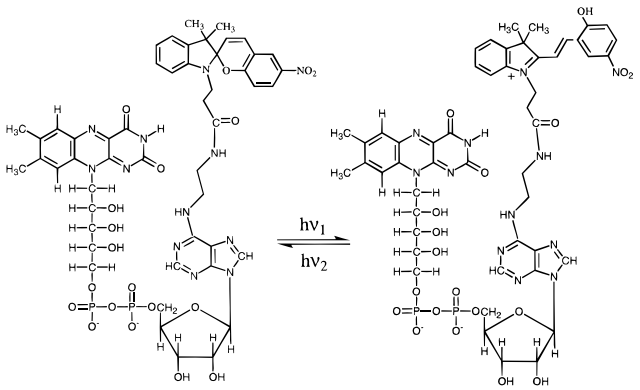
Photostimulation of enzyme functions represents a basic feature for the application of biocatalysts in bioelectronic and optoelectronic devices.[1,2] Photoswitchable activation and deactivation of redox proteins provides a means for the amperometric transduction and amplification of recorded optical signals.[3] A monolayer electrode consisting of glucose oxidase, modified by nitrospiropyran photoisomerizable units, revealed photoswitchable electrocatalytic properties. The electrode was applied for reversible amperometric transduction of optical signals recorded by the enzyme monolayer.[4] Other methods to photostimulate the activities of redox proteins applied photoisomerizable monolayer-modified electrodes[5] or photoisomerizable electron mediators[6] to control by the light electrical communication between the biomaterials and electrode surfaces. Here we wish to report on a novel method to tailor photoswitchable redox biocatalysts by reconstitution of apo-flavoenzymes with a nitrospiropyran-modified flavin adenine dinucleotide, FAD, cofactor.

The presently available methodology to design photoswitchable enzymes by chemical modification of the protein with photoisomerizable components is limited as a result of the random, nonspecific, substitution of the biopolymers.[2,7] This yields the modification of protein positions which are remote from the active site, and hence the structural perturbations of the catalytic site are minimal. As a result, incomplete photochemical deactivation of the enzyme OFF state is always observed. Furthermore, the nonspecific modification of the enzyme by photoisomerizable groups excludes the possibility to structurally correlate the switched-off biocatalytic functions of the enzyme and the photoisomer configuration with the protein structure. The reconstitution of an apo-flavoenzyme, i.e., glucose oxidase, with a photoisomerizable unit-modified FAD cofactor provides a general means for site-specific modification of the redox enzyme. Targeting of the photoisomerizable component into the protein by means of specific association of the FAD cofactor yields a defined configuration of the light-active unit in close proximity to the enzyme redox center.



SP-FAD, (**1a**) MRH$^+$-FAD, (**1b**)

The nitrospiropyran-modified FAD, SP−FAD, **1a**, was prepared by coupling[8] of N^6-(2-aminoethyl)-FAD, **2**,[9] with 1′-(*β*-carboxyethyl)-3′,3′-dimethyl-6-nitro-[2*H*-1]-spiro[benzopyran-2,2′-indole], **3**.[10] The nitrospiropyran-modified FAD, **1a**, reveals reversible photoisomerizable properties. Illumination of **1a**, 360 nm < λ < 380 nm, yields the nitromerocyanine−FAD isomer state, **1b**, MRH$^+$−FAD, exhibiting an absorption band in the region of 320−560 nm that corresponds to the overlapping bands of the MRH$^+$ (520 nm) and FAD (355, 460 nm) chromophores. Further irradiation of the MRH$^+$−FAD solution, λ > 475 nm, yields a yellow solution exhibiting the FAD absorbance band at λ = 360 and 447 nm and the characteristic SP-absorbance in the UV region. The photoisomerization between the SP−FAD and MRH$^+$−FAD is reversible. The cyclic voltammograms of SP−FAD and MRH$^+$−FAD reveal the characteristic reversible two-electron redox process of the FAD cofactor unit at $E°$= −0.50 V vs SCE, pH = 7.0. The redox potential of the FAD unit is not influenced by the linked photoisomer component and is identical for the SP−FAD, **1a**, and MRH$^+$−FAD, **1b**.

Apo-glucose oxidase,[11] apo-GOD, was reconstituted[12] with the photoisomerizable FAD cofactor **1a**.[13] The loading of the SP−FAD cofactor corresponds to 2 indicating that the two enzyme subunits are occupied by the photoisomerizable semisynthetic cofactor. The reconstituted enzyme exhibits photoisomerizable properties. Illumination of **1a**-reconstituted GOD, 360 nm < λ < 380 nm, yields **1b**-GOD, and further irradiation of **1b**-GOD, λ > 475 nm, regenerates **1a**-GOD.

Ferrocene derivatives were reported as effective electron transfer mediators for electrobiocatalyzed oxidation of glucose by GOD.[14,15] Accordingly, the bioelectrocatalyzed oxidation of glucose (5×10^{-2} M, saturation conditions) was examined

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(8) Coupling was performed in the presence of 1-ethyl-3-(dimethylaminopropyl)carbodiimide, EDC, in HEPES buffer, 0.1 M, pH = 7.5, while shaking for 3 h at 30 °C. The product (**1a**) was purified by thin layer chromatography on SiO_2; isopropanol:H_2O (7:3) was used as eluent.

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(12) Reconstitution was accomplished by vigorous shaking, for 24 h, of apo-glucose oxidase and **1a** (molar ratio 1:10) in a phosphate buffer, 0.1 M, pH = 7.0. The product was dialyzed against phosphate buffer, 0.1 M, pH = 7.0 for 30 h.

(13) The activity of **1a**-GOD is 80% of native GOD in the presence of oxygen as oxidant. The enzyme was assayed by analyzing the **1a**-GOD generated H_2O_2 using *o*-dianisidine and peroxidase as an indicator. For oxygen as co-substrate, the enzymes **1a**-GOD and **1b**-GOD differ only by ca. 10% in their activities. Thus, **1a**-GOD does not sterically perturb the O_2 penetration path.

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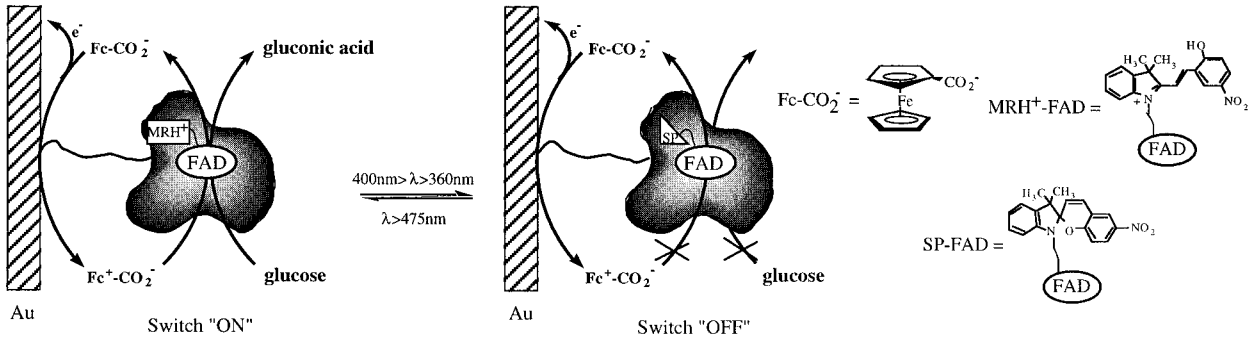

Figure 1. Photostimulated bioelectrocatalyzed oxidation of glucose by the reconstituted photoisomerizable GOD monolayer electrode in the presence of ferrocene carboxylic acid as diffusional electron mediator.

at different concentrations of ferrocene carboxylic acid in the presence of **1a**-GOD or **1b**-GOD. The resulting electrocatalytic anodic currents were analyzed in terms of the Michaelis−Menten model. The two photoisomer states of the enzyme reveal similar I_{max} ($= 6.9 \times 10^{-6}$ A) values, whereas the K_m values of the two enzyme states differ substantially (for **1a**-GOD, $K_m = 0.8$ mM and for **1b**-GOD, $K_m = 0.3$ mM). These results imply that the electron transfer rate of the oxidation of the FAD cofactor by the ferrocenylium cation electron mediator is of similar effectiveness in the two photoisomer states of the reconstituted enzyme, but the interactions of the electron mediator with the protein to attain the appropriate configuration for electron transfer differ for **1a**-GOD and **1b**-GOD. That is, in the nitrospiropyran−FAD-reconstituted GOD, **1a**-GOD, the penetration path of the ferrocenylium cation into the protein, to yield the appropriate steric configuration that allows oxidation of FAD, is perturbed. Similar conclusions were recently reported[16] for the chiroselective electrical communication between GOD and chiral ferrocene electron mediators.

The kinetic analysis of the electrobiocatalyzed oxidation of glucose by **1a**-GOD and **1b**-GOD allows us to select the ferrocene carboxylic acid concentration, where maximal differences in recognition of the electron mediator by the enzyme photoisomer states occur. Furthermore, at these conditions, the reconstituted enzyme can be assembled onto an electrode acting as an optoelectronic device for the amperometric transduction of recorded optical signals. **1a**-GOD was assembled as a monolayer onto a rough Au electrode.[17] A cystamine monolayer assembled on the Au electrode was reacted with glutaric dialdehyde, and subsequently, **1a**-GOD was covalently linked to the monolayer (surface coverage of the enzyme $\approx 1 \times 10^{-11}$ mol·cm^{-2}), Figure 1. Figure 2A shows the cyclic voltammograms obtained upon electrobiocatalyzed oxidation of glucose by the photoisomerizable enzyme monolayer electrode and in the presence of ferrocene carboxylic acid, 5×10^{-5} M, as diffusional electron mediator. With the **1a**-GOD monolayer electrode, a low electrocatalytic anodic current is observed (curve a). Upon photoisomerization of the monolayer to the **1b**-state, an 11-fold enhancement in the electrocatalytic anodic current is observed (curve b). Thus, the nitromerocyanine−FAD-reconstituted enzyme, **1b**-GOD, reveals a substantially higher electrocatalytic activity and represents a switched-on biocatalyst, where the **1a**-GOD exhibits decreased biocatalytic properties and represents a switched-off enzyme. By reversible photoisomerization of the monolayer electrode between (**1a**)-GOD and (**1b**)-GOD states, cyclic amperometric transduction of the recorded optical signals is accomplished, Figure 2B. Cyclic low or high amperometric responses are detected upon photoisomerization of the monolayer through the states **1a**-GOD and **1b**-GOD, as a result of photoswitchable electrical communication between the biocatalyst and electrode, Figure 1.

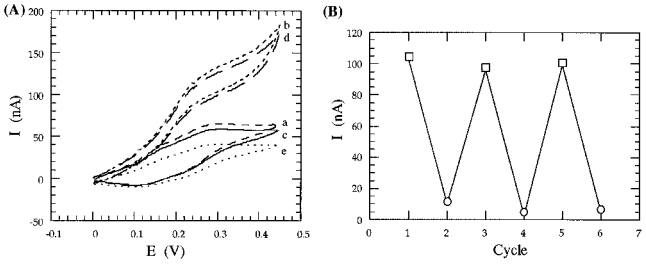

Figure 2. (A) Cyclic voltammograms of the photoisomerizable monolayer electrodes in the presence of glucose, 5×10^{-2} M, and ferrocene carboxylic acid, 5×10^{-5} M: (a) and (c) with the **1a**-GOD electrode; (b) and (d) with the **1b**-GOD electrode; (e) with **1a**-GOD electrode and ferrocene carboxylic acid, 5×10^{-5} M, in the absence of glucose. All measurements were recorded in phosphate buffer, 0.01 M, pH = 7.3, and sodium sulfate, 0.1 M, 38 °C, under an inert Ar atmosphere. A Au foil electrode, geometrical area 0.4 cm^2 (roughness coefficient ~20) was used, scan rate 5 mV·s^{-1}; SCE was employed as reference. (B) Cyclic amperometric transduction of optical signals recorded by the reconstituted photoisomerizable GOD electrode: (□) **1b**-GOD electrode; ○ **1a**-GOD electrode. Net electrocatalytic anodic currents at $E = 0.4$ V are presented. Currents were elucidated by subtraction of the background current of the electron mediator (curve e) from the cyclic voltammogram of the **1a**-GOD or **1b**-GOD electrodes, respectively (curves a or b).

In conclusion, we demonstrate a novel method to organize photoswitchable redox proteins for amperometric transduction of optical signals. The method consists of reconstitution of a flavo-apoenzyme, apo-GOD, with a synthetic FAD analog modified by a nitrospiropyran photoisomerizable unit. The protein-implanted photoisomerizable group delicately controls the protein structure in the FAD cofactor surrounding. The site-specific modification of the enzyme by the photoisomerizable units through the application of the reconstitution methodology represents a major advance in designing photoswitchable enzymes. It enables further structural characterization of the biocatalyst and elucidation of the steric perturbations of the protein stimulated by photoisomerizable components.

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