DOI: 10.1002/anie.200902191

Direct Electron Transfer

Deglycosylation of Glucose Oxidase for Direct and Efficient Glucose Electrooxidation on a Glassy Carbon Electrode**

*Olivier Courjean, Feng Gao, and Nicolas Mano**

Glucose oxidase (GOx) from *Aspergillus niger*, a dimeric approximately 160 kDa flavoenzyme that is glycosylated to 16–25 wt%, catalyzes the dioxygen-oxidation of glucose to gluconolactone and H_2O_2. Each of its halves contains a tightly bound and deeply buried flavin adenine dinucleotide (FAD) center about 15 Å below the protein surface.[1] These centers are reduced by glucose to $FADH_2$. $FADH_2$ is reoxidized to FAD either by O_2, which is reduced to H_2O_2, or by the oxidizing member of a redox couple. In electrochemical glucose sensors, on which about 2400 articles have been published (SciFinder Scholar 2008), the electrooxidation of either H_2O_2 or of the reduced member of the redox couple is usually monitored.[2] Because of the failure of reported attempts to electrooxidize GOx-$FADH_2$ directly on simple glassy carbon or gold surfaces, redox mediators, carbon-nanotube-modified electrodes, or conductive nanoparticles are used to mediate the transport of electrons from $FADH_2$ to the electrodes.[3–7] The one exception, whereby electrons were transferred directly from GOx-$FADH_2$ to graphite, formed the subject of a patent application filed in 1989 by Kalisz and Künnecke.[8] The inventors reported that a deglycosylated glucose oxidase from *Penicillium amagasakiense* immobilized on a graphite electrode oxidized glucose at 0 V versus Ag/AgCl.

Herein we show that the deglycosylation of glucose oxidase from *Aspergillus niger* yields a fully active deglycosylated GOx (dGOx). When a monolayer of the new dGOx was immobilized on a vitreous carbon electrode, the electrooxidation of glucose already started at the unprecedented reducing potential of −490 mV versus Ag/AgCl, the redox potential of the adsorbed enzyme. At −200 mV (Ag/AgCl) and a glucose concentration of 45 mM, glucose was electrooxidized directly with the production of a current density of 235 $\mu A\,cm^{-2}$ (Figure 1). The electron-transfer turnover rate was 1300 s^{-1}: about twice as fast as the turnover rate of GOx when the $FADH_2$ moieties were oxidized by air.

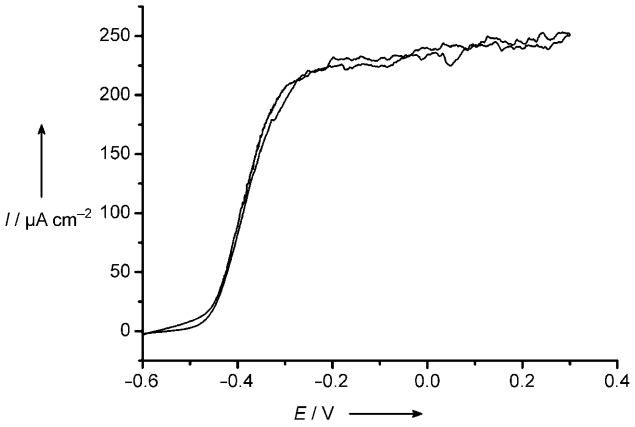

Figure 1. Direct electrooxidation of glucose (45 mM) on a monolayer of deglycosylated *Aspergillus niger* glucose oxidase adsorbed on a glassy carbon electrode (20 mM phosphate buffer, pH 7.4, scan rate: 5 $mV\,s^{-1}$, 500 rpm, argon atmosphere).

For native glycosylated GOx, the failure to transfer electrons from GOx-$FADH_2$ to electrodes has been attributed to the slow transfer of electrons across the 13–15 Å distance between the $FADH_2$ centers and the electrode surface.[1] To shorten this distance and increase the rate of electron transfer, we chose to deglycosylate GOx. Removal of the sugar residues can be controlled in such a way that it is possible to generate either partially or nearly fully deglycosylated proteins. It was shown previously that partial deglycosylation of the GOx did not shorten this distance sufficiently to enable the direct electrooxidation of the protein-buried $FADH_2$ centers.[9]

The nearly fully deglycosylated GOx was obtained by a procedure adapted from a protocol described by Kalisz et al.[10] The purified GOx was incubated for 72 h with α-mannosidase and endoglycosidase H in a 100 mM phosphate buffer at pH 5.1 and 37 °C. This treatment cleaves the oligosaccharides at N-glycosylation sites after the first *N*-acetylglucosamine residue, as seen in the X-ray crystal structure of the GOx as the *N*-asparagine-bound acetylglucosamine.[11] The resulting dGOx was purified by fast protein liquid chromatography (FPLC) on a cation-exchange column. Cleavage of the N-linked oligosaccharides was confirmed by denaturing SDS-PAGE followed by mass spectrometry (see the Supporting Information).

Denaturing SDS-PAGE decomposed the dimeric GOx into the monomeric GOx. Cleavage of the oligosaccharides decreased the mass of the GOx monomer from (78 ± 6) kDa to (69 ± 4) kDa for dGOx, a higher value than the mass of

[*] Dr. O. Courjean,[+] Dr. F. Gao,[+] Dr. N. Mano
Université de Bordeaux
Centre de Recherche Paul Pascal (CRPP)-UPR 8641
Avenue Albert Schweitzer, 33600 Pessac (France)
Fax: (+33) 5-5684-5600
E-mail: mano@crpp-bordeaux.cnrs.fr

[+] These authors contributed equally to this work.

[**] This research was financed by a European Young Investigator Award (EURYI) and la Région Aquitaine. We thank Prof. Heller for helpful discussions, Hassan Saadoui for help with the AFM experiments, and Maryse Maugey for help with the DLS experiments.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200902191.

63.3 kDa calculated from the primary amino acid sequence of the fully deglycosylated enzyme. This 6 kDa difference can be attributed to the reduced accessibility of the glycosylation site at the interface of the two subunits. It corresponds to the remaining asparagine-bound acetylglucosamine residue and a large carbohydrate chain that links the tip of the FAD-binding lid of one subunit with the second subunit of the dimer.[11] To confirm that no FAD had leached, the protein-FAD concentration was calculated from the UV/Vis absorption at 452 nm, and the protein concentration from its absorption at 280 nm. The specific activity, k_{cat} value, and K_m value of the native and the 138 kDa deglycosylated GOx were determined by measuring the steady-state reaction rate as a function of glucose concentration (between 2 μM and 150 mM). In agreement with a previous study,[12] there was little difference between the activities of the GOx and the dGOx (specific activity of the GOx and dGox: approximately 65 U nmol^{-1}; $k_{cat} \approx 1350\ s^{-1}$; $K_m \approx 27$ mM; see Table 1 in the Supporting Information).

Dynamic light scattering measured at pH 5 and 37 °C showed a hydrodynamic diameter of (89 ± 4) Å for a sphere-modeled native GOx and a hydrodynamic diameter of (76 ± 3) Å for the corresponding sphere-modeled dGOx. In the crystal structure (at a resolution of 2.3 Å),[11] the monomeric partially deglycosylated dGOx is a $60 \times 52 \times 37$ Å^3 compact spheroid, and the dimer is a $60 \times 52 \times 77$ Å^3 compact spheroid, from which we conclude that dGOx is dimeric. In AFM images, the line scan revealed globular structures, the height of which matched those reported previously (see the Supporting Information).[13,14] Most significantly for explaining (see below) the uniquely rapid electron transfer from the dGOx-$FADH_2$ centers to the vitreous carbon, we also observed the reported butterfly shape of the adsorbed GOx.[14]

Figure 2 shows cyclic voltammograms of glassy carbon electrodes on which purified GOx and dGOx were adsorbed. The voltammograms, attributed to the FAD/$FADH_2$ cofactors in GOx and dGOx, exhibit a symmetrical wave at −490 mV versus Ag/AgCl with little separation between the oxidation and reduction peaks. This pattern is indicative of a reversible surface-bound redox couple. The potential is about 250 mV more reducing than the one-electron potentials reported for GOx-FAD[15] and can be explained by a strong interaction with the adsorbing vitreous carbon, or by a different reaction of the cofactor, such as its two-electron reduction/oxidation. The peak height varies linearly with the scan rate; this relationship shows that the redox couple is surface-confined. The width of the peak at half height, E_{whm}, is 52 mV, which is close to the theoretical width of 47.2 mV for an ideal Nernstian two-electron-transfer reaction at 37 °C. The peaks of the native GOx are about five times smaller than those of the dGOx; thus, the FAD/$FADH_2$ centers in dGOx are electrically much better connected to the electrode than it is in GOx. This point is illustrated by the rates of electron transfer of 0.2 s^{-1} for GOx and 1.58 s^{-1} for dGOx, as calculated by using the Laviron formalism.[16]

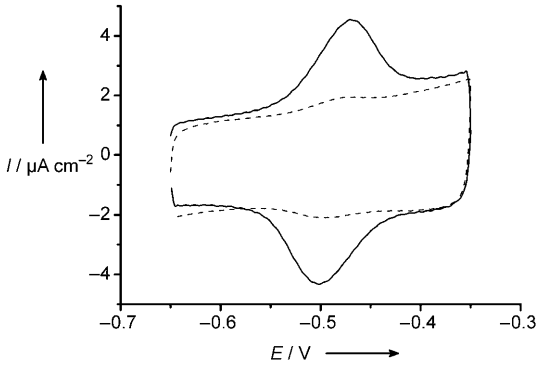

Figure 2. Cyclic voltammograms of GOx (dotted line) and dGOx (solid line) adsorbed on glassy carbon electrodes (20 mM phosphate buffer, pH 7.4, 37 °C, scan rate: 20 mV s^{-1}, argon atmosphere).

The observed current density of 235 μA cm^{-2} for the dGOx monolayer on vitreous carbon (Figure 1) corresponds to half the value of the current density (460 μA cm^{-2}) reported by Xiao et al. for a GOx monolayer in which electrons were relayed by 1.4 nm diameter gold nanoparticles between FAD/$FADH_2$ and a gold electrode.[3] In that study, the electron turnover rate for the glucose reduction of FAD and electrooxidation of $FADH_2$ was estimated at approximately 5000 s^{-1}. This value is about seven times higher than the turnover rate of GOx when the $FADH_2$ centers are oxidized by air. In the present study, we also calculated a high turnover rate of 1300 s^{-1} from the anodic-current-density plateau and the surface coverage of the enzyme molecules. Whereas high turnover was observed by Xiao et al. only at about +0.8 V versus Ag/AgCl, we already observed high turnover in dGOx at −0.2 V versus Ag/AgCl, without mediation by gold nanoparticles. The onset of glucose electrooxidation, which only occurred at +0.25 V versus Ag/AgCl in the study by Xiao et al., occurred at −490 mV versus Ag/AgCl in our study.

We propose that in mixtures of globular and butterfly-shaped dGOx, the adsorption of the butterfly-shaped dGOx on vitreous carbon is thermodynamically preferred. This adsorption has three effects: first, it stabilizes the enzyme; second, it establishes an electrical contact between both FAD/$FADH_2$ centers and the carbon surface and thus effectively decreases the distance between the cofactor and the carbon surface; and third, it decreases the redox potential of the two FAD/$FADH_2$ centers to −490 mV versus Ag/AgCl. For the first time, we showed that glucose is electrooxidized rapidly at an unprecedented reducing potential when the reaction is catalyzed by the deglycosylated *Aspergillus niger* glucose oxidase on glassy carbon. The electron-transfer turnover rate (1300 s^{-1}) is about twice as fast as the turnover rate for native GOx when O_2 is the acceptor. In addition to possible applications in biofuel cells and glucose sensors, we believe that this study may be extended to others enzymatic systems and offers a new strategy to circumvent poor electrical contact between enzymes and electrode surfaces.

Received: April 23, 2009
Published online: June 30, 2009

Keywords: biosensors · direct electron transfer · electrochemistry · enzymes · glucose oxidases

[1] G. Wohlfahrt, S. Witt, J. Hendle, D. Schomburg, H. M. Kalisz, H.-J. Hecht, *Acta Crystallogr. Sect. D* **1999**, *55*, 969.
[2] A. Heller, B. Feldman, *Chem. Rev.* **2008**, *108*, 2482.
[3] Y. Xiao, F. Patolsky, E. Katz, J. F. Hainfield, I. Willner, *Science* **2003**, *299*, 1877.
[4] F. Patolsky, Y. Weizmann, I. Willner, *Angew. Chem.* **2004**, *116*, 2165; *Angew. Chem. Int. Ed.* **2004**, *43*, 2113.
[5] D. Ivnitski, K. Artyushkova, R. A. Rincon, P. Atanassov, H. R. Luckarift, G. R. Johnson, *Small* **2008**, *4*, 357.
[6] G. Liu, M. N. Paddon-Row, J. J. Gooding, *Electrochem. Commun.* **2007**, *9*, 2218.
[7] While this Communication was in press, a different strategy to increase the direct electron transfer between GOx and an electrode surface was reported, with a rate constant of 350 s^{-1}: S. Demim, E. A. H. Hall, *Bioelectrochemistry* **2009**, in press.
[8] H. M. Kalisz, W. Künnecke, DE 3925140A1, Germany, **1989**.
[9] D. M. Fraser, S. M. Zakeeruddin, M. Gratzel, *Biochim. Biophys. Acta Bioenerg.* **1992**, *1099*, 91.
[10] H. M. Kalisz, H. J. Hecht, D. Schomburg, R. D. Schmid, *J. Mol. Biol.* **1990**, *213*, 207.
[11] H. J. Hecht, H. M. Kalisz, J. Hendle, R. D. Schmid, D. Schomburg, *J. Mol. Biol.* **1993**, *229*, 153.
[12] H. M. Kalisz, H. J. Hecht, D. Schomburg, R. D. Schmid, *Biochim. Biophys. Acta Lipids Lipid Metab.* **1991**, *1080*, 138.
[13] M. Wang, S. Bugarski, U. Stimming, *J. Phys. Chem. C* **2008**, *112*, 5165.
[14] D. Losic, J. G. Shapter, J. J. Gooding, *Langmuir* **2002**, *18*, 5422.
[15] M. T. Stankovich, L. M. Schopfer, V. Massey, *J. Biol. Chem.* **1978**, *253*, 4971.
[16] E. Laviron, *J. Electroanal. Chem.* **1979**, *101*, 19.

