Direct electron transfer involving a large protein: glucose oxidase†

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Electrochemical behavior of glucose oxidase fixed in the bulk of a graphite paste electrode, with no additives available to play the role of mediator or redox activator, shows the oxido reduction of the prosthetic FAD group that is still attached to the apoenzyme with no loss of enzyme activity.

Since Updike and Hicks[1] described the first enzyme electrode in 1967, a great amount of work has been devoted to the study of the contacts and exchange reactions between enzymes and electrodes. The association between an electrode material and biological macromolecules, such as proteins, and the study of their interactions may lead to useful information. At first sight, this combination could be used as a model for the investigation of the intrinsic redox behaviors of proteins. Moreover, the "protein electrode complex" may worthily be used as an analogue of the protein–protein complexes involved in a large number of physiological electron transfer reactions. This implies that the communication between the protein and the electrode material should be as direct and fast as in physiological systems. On the other hand, an efficient exchange between the redox proteins and the environment which uses them is essential in order to make the best benefit of their peculiar properties in biotechnological applications such as biosensors or biocatalysis.

Until recently, it was believed to be difficult to achieve direct and reversible electron transfer between redox proteins and electrodes. Extensive research has been done over the last twenty years to study the electrochemical behaviour of small redox proteins.[2,3] For several metalloproteins such as cytochrome c, flavodoxin, ferredoxin, rubredoxin or plastocyanin, direct electron transfer was observed. Electrochemical studies with large redox enzymes have proved to be less successful. A recent review[4] on fundamentals and analytical applications of enzyme-catalysed direct electron transfer related that a number of enzymes were found to be capable of interacting directly with an electrode while catalysing the corresponding enzymatic reaction, whereas controversial results were reported on the direct electrocatalysis of glucose oxidase (GOD). Several studies indicated that no enzymatic activity associated with the observed electron transfer has been detected for GOD,[5,6] and that its electrocatalytic activity decreases rapidly while catalysing glucose electrooxidation.[7] This suggests that the enzyme has undergone important structural transformations during its fixing to the surface of the electrode and that the observed electron transfer does not concern the enzyme itself, or that the activity of the protein has been destroyed following the transfer of electrons. In the two cases the electrochemical signal observed cannot be directly linked to the enzymatic activity of the protein nor is it indicative of the oxidation state of the enzyme.

† *Supplementary data available:* cyclovoltammetric data. Available from BLDSC (No. SUP 57580, 4 pp.). See Instructions for Authors, 1999, Issue 1 (http://www.rsc.org.njc).

Previous studies on the direct electrochemistry of enzymes, in the absence of mediators or activators, have been attempted using mercury or conventional solid electrodes on the surface of which the enzyme was adsorbed or covalently attached.[8,9] Although the carbon paste electrode is now widely used, few attempts have been made to exploit the intimate association of proteins with the transducer in order to observe the direct electrochemical communication between them. Yabuki *et al.*[10] reported that the cyclic voltammogram of the carbon paste electrode containing GOD modified with polyethylene glycol (PEG-GOD) shows oxidation and reduction peaks respectively at −0.3 and −0.45 V *vs.* Ag–AgCl. They also reported that in contrast no redox current peak was detected around these potentials when unmodified GOD was used in the enzyme electrode. In the present study, we report an example of direct electron transfer involving a large protein such as glucose oxidase with no loss of its apparent enzyme activity. This electron transfer is directly linked to the enzyme that is fixed in the bulk of a graphite paste electrode in the absence of additives or modifiers.

Cyclic voltammetry using a GOD modified electrode [GOD-electrode] in deaereted 0.1 M phosphate buffer solution, pH = 7.2, without previous chemical or electrochemical pre-treatment of the electrode surface shows a well defined and reproducible electrochemical response [Fig. 1(a)]. A nearly reversible redox system is observed ($E^{p}_{red} = -0.46$, $E^{p}_{ox} = -0.38$ V *vs.* SCE; $\Delta Ep = 80$ mV and $i^{p}_{ox}/i^{p}_{red} = 0.65$ for scan rate of 0.5 mV s^{-1}). Cyclic voltammetry using graphite paste electrode with no GOD in its bulk shows no response in phosphate buffer in the presence or in the absence of GOD in solution.

In order to determine whether the electron transfer is provoked by the reduction of FAD linked to the protein or by the reduction of free FAD molecules released from the enzyme during the carbon paste electrode preparation, a FAD modified graphite paste electrode [FAD-electrode] was prepared in

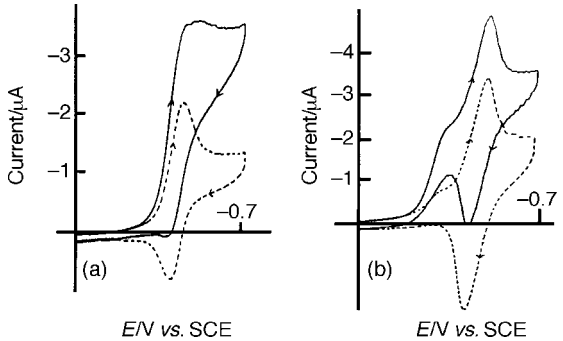

Fig. 1 Cyclic voltammogram of (a) the GOD and (b) the FAD-electrode in 0.1 M phosphate buffer, pH 7.2, (– – – –) in the absence and (———) in the presence of oxygen. Electrode area, 0.07 cm^2. Scan rate, 0.5 mV s^{-1}.

the same way as the GOD-electrode. The FAD-electrode shows, in deaereted phosphate buffer solution (0.1 M, pH = 7.2), a different behaviour to that observed with the GOD-electrode. Its cyclic voltammogram [Fig. 1(b)] displays a reproducible electrochemical signal showing a reversible system at −0.50 V *vs.* SCE for the cathodic peak and −0.44V *vs.* SCE for the anodic one as well as a cathodic prewave at around −0.4 V *vs.* SCE. When a mixture of GOD and free FAD are included in the carbon paste electrode, the cyclic voltammogram in the presence or in the absence of oxygen is the sum of the two independent voltammograms.

Three different sources may be attributed to the electron transfer observed with the GOD-electrode: a direct transfer to the active enzyme, an electron transfer involving the detached FAD or the oxido reduction of the FAD that is still attached to a denatured enzyme. It has been shown[11] that in the latter case, even when FAD is not divorced from the apoenzyme, the cyclic voltammogram is completely consistent with the one of free FAD. Fig. 1 clearly indicates that cyclic voltammograms of the GOD and FAD-electrode are different implying that in the GOD-electrode, FAD groups are still attached to the active enzyme.

Investigations concerning the oxido reduction of FAD have been made using electrochemical techniques.[12–14] Cyclic voltammetry indicates that FAD is strongly adsorbed on the surface of the electrode (mercury, graphite, gold, platinum) and shows a pair of cathodic and anodic peaks corresponding to a two-electron transfer process. A cathodic prepeak is also observed depending on the pH and the electrode material. The overall reduction of FAD implies two electrons and two protons are involved leading to the dihydroflavin compound $FADH_2$:

$$FAD + 2e^- + 2H^+ \leftrightarrows FADH_2 \quad (1)$$

The electrochemical behaviour of entrapped FAD in a carbon paste matrix is also consistent with this scheme at least at neutral pH.

It has been reported[15] that the prosthetic FAD group can be easily detached from the apoenzyme when GOD is reacted with a highly concentrated solution of urea. We immersed the GOD-electrode in a 6 M urea solution for 30 s and recorded the cyclic voltammogram in phosphate buffer. Such a treatment leads to exactly the same voltammogram as the one obtained with the FAD-electrode. This indicates that the cathodic and anodic peaks observed with the GOD-electrode are likely due to the oxido reduction of the prosthetic FAD group which is still attached to the apoenzyme.

In order to validate that the electron transfer involved in the GOD-electrode does not result from detached FAD the two modified electrodes were submitted to the same reactant, namely O_2, and their respective behaviours were compared. Fig. 1 shows that the two electrodes are affected when O_2 is present in the buffer solution but they behave differently. For the GOD-electrode the reduction peak increases while the oxidation peak decreases, particularly at slow potential scan rates. For the FAD-electrode only the cathodic prepeak is strongly affected in the presence of O_2 indicating that the two species generated after the electron transfers involving the GOD and the FAD-electrode are different.

By comparison with the mechanism recognised for the reduction of free FAD, the electrochemical behaviour of the GOD-electrode in the absence of oxygen is the following:

$$GOD\text{-}FAD + 2e^- + 2H^+ \leftrightarrows GOD\text{-}FADH_2 \quad (2)$$

In the presence of oxygen, the reduced enzyme is oxidised very quickly at the surface of the electrode.

$$GOD\text{-}FADH_2 + O_2 \rightarrow GOD\text{-}FAD + H_2O_2 \quad (3)$$

The catalytic regeneration of the enzyme in its oxidised form causes the loss of reversibility and the increase in size of the cathodic peak. This is still visible even for relatively high potential scan rates (1 V s^{-1}) because of the very important reactivity of the enzyme in its reduced form towards the oxygen.

Another important difference between the GOD and the FAD-electrode is observed when glucose is added to the non-deaerated buffer solution. Glucose does not affect the FAD-electrode behaviour, while the reduction peak observed with the GOD-electrode decreases when the glucose concentration increases until it attains the height of the peak observed in the absence of O_2 (Fig. 2). This is concordant with a competitive reaction occurring at the vicinity of the electrode surface: GOD uses the dissolved oxygen to enzymatically oxidise the glucose, this leads to the depletion of the oxygen from the surface of the electrode making its reaction with GOD-$FADH_2$ [reaction (3)] less favourable. A Mickaëlis type curve is obtained when current is plotted against glucose concentration showing for the enzyme an apparent K_M of 4.4 mM. The maximum rate, Vm, of the reaction is limited by the oxygen concentration. The plot is linear between 0 and 0.55 mM of glucose. This competitive reaction shows that GOD is still active at the surface of the electrode and does not suffer during the electrode preparation. This activity is not modified even when repetitive cyclic voltammetry of the GOD-electrode is run for a long time before adding the glucose.

Under anaerobic conditions, enhancement of the anodic peak of the GOD-electrode, on addition of glucose, was observed using cyclic voltammetry. This could result from the catalytic regeneration of the reduced form of GOD by glucose in the absence of oxygen. The enzymatic activity of the electrode surface was also confirmed by the increase of the oxidation current after the addition of glucose to the deaerated buffer solution when the potential of the GOD-electrode is set at 0.2 V *vs.* SCE. Fig. 3 shows the maximum increased current at each glucose concentration. The increased current is proportional to the glucose concentration and the apparent K_M is equal to 5.5 mM. Again the GOD-electrode, set at 0.2 V *vs.* SCE, shows anodic signals when used in a flow injection analysis mode (FIA). The heights of the signals are proportional to the glucose concentration. At this potential no signal is observed when hydrogen peroxide or fructose solutions are injected or when the FAD-electrode is used or glucose solutions are injected.

The electrochemical behaviour of the GOD-electrode in the absence and in the presence of glucose may account for a direct electron transfer between the electrode and the enzyme. Additional experiments, including FIA assays, on the use of such a direct electron transfer for the design of new biosensors

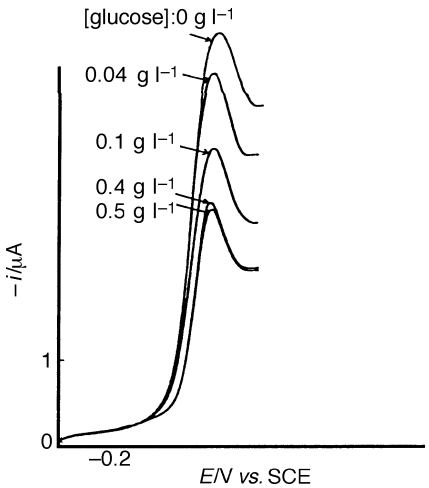

Fig. 2 Cyclic voltammogram of the GOD-electrode in the presence of oxygen and in increasing amounts of glucose. Electrode area 0.07 cm^2. Scan rate 2 mV s^{-1}.

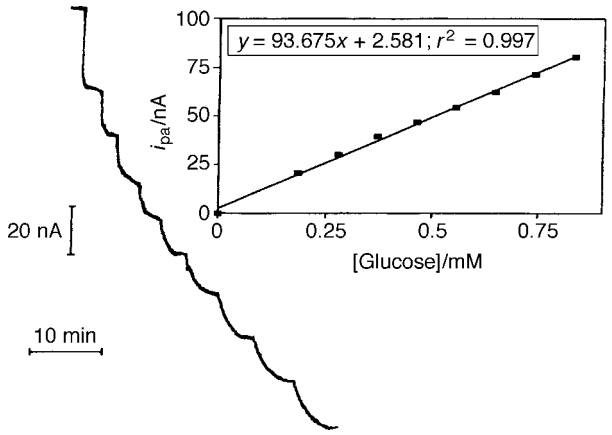

Fig. 3 The response of the GOD-electrode to the addition of glucose solution. Insert: plot of the current *vs.* concentration of glucose/mmol dm^{-3}.

and for a better understanding of redox reactions of proteins will be the topic of future accounts from this laboratory.

Experimental

Glucose oxidase (GOD) from *Aspergillus niger* [EC.1.1.3.4 was purchased from Biozyme Ltd (154 U mg^{-1}, $M = 160\,000$)]. FAD disodium salt ($M = 829.5$) (Sigma, F-6625), glutaraldehyde (Sigma, G-6257) and bovine serum albumin (BSA, Sigma, A-2153) were obtained from Sigma Chemical Ltd. Castor oil (cat. No 83905) was obtained from Fluka Chemical Ltd. Graphite powder (cat. No 16858) was purchased from Le Carbone Lorraine.

Cyclic and linear sweep potential voltammetry measurements were performed with an Amel potentiostat, model 472, connected to a BD90 XY recorder. A three-electrode system with a SCE reference electrode and a platinum wire as auxiliary electrode was used. All potential values are given *versus* SCE. The working electrodes are either GOD or FAD carbon paste modified electrodes. The geometric area of the working carbon paste electrode is 7 mm^2. All experiments were conducted at room temperature.

The carbon paste electrodes used were prepared using GOD and BSA, glutaraldehyde being used to chemically cross-link the proteins *via* the amino groups present. The standard method is described here: the active enzyme powder (1 g quantity) was prepared by dissolving 50 mg of BSA in 0.8 ml of phosphate buffer (0.01 M, pH = 7.2), after which 160 mg of glucose oxidase were added. Following the dissolution of the enzyme, 0.29 g of a 0.25% w/w glutaraldehyde solution was added to form a cross-linked gel. The enzyme mixture was stirred for 10 min, after which 0.79 g of graphite powder was slowly added and further stirred to allow homogenisation. The mixture was dried either in a vacuum dessicator or lyophilised at −20 °C in a freeze drier. The dried powder was crushed in a mortar and sieved (80 μm) and then mixed with castor oil: 23.66% (m/m) of oil. The resulting paste was then packed into a plastic tube (cartridge) with an internal diameter of 0.3 cm forming the carbon paste working electrode. A graphite rod was inserted into one end of the cartridge to form an electrical contact with the sensing element and the measuring device. Before use, a small amount of carbon paste is expelled from the plastic cartridge and the surface of the working electrode is polished on a paper and dipped in the electrolytic solution.

Variants of this method of preparation have been equally achieved. Thus BSA and the enzyme have been dissolved in distilled water instead of the buffered solution, BSA has not been incorporated in the electrode or glutaraldehyde has not been used. In all cases the electrochemical characteristics of consequent electrodes were identical, only the stability of the surface of the electrode was best when the standard method described previously was used.

In order to load a FAD modified carbon paste electrode with an equivalent amount of protein as the GOD-electrode, 208.5 g of BSA were dissolved in 0.8 ml phosphate buffer solution (0.01 M, pH = 7.2), after which 1.5 mg of FAD were added (this approximately represents the ratio of FAD present in the 160 mg of GOD), followed by the addition of 0.29 g of a 0.25% w/w glutaraldehyde solution. The mixture was stirred for 10 min, after which 0.79 g of graphite powder was slowly added and stirred until homogenisation. The resulting mixture was dried, mixed with castor oil (23.66% m/m) and packed into the plastic cartridge to be used as the working electrode in the same way as for the GOD-electrode.

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