The Direct Electron Transfer of Myoglobin Based on the Electron Tunneling in Proteins

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The electron tunneling of the protein−polypeptide interactions was observed in the study of direct electron transfer of the myoglobin (Mb) on the electrode surface. The Mb was selected as a redox active protein and gelatine was selected to couple with Mb to form an electron tunneling. The electrochemical results indicated the presence of the electron tunneling and the direct electron transfer. The circular dichroism spectra suggested that the β-sheet chain of gelatine could interact with α-helical chain to form an electron tunneling to promote the protein direct electrochemistry. The SDS−PAGE results proved that the electron tunneling between Mb and gelatine was noncovalent hydrogen bonds. The immobilized Mb showed a couple of quasi-reversible redox peaks with a formal potential of −0.37V (vs SCE) in 0.1 M pH 7.0 PBS. The modified electrodes displayed a rapid amperometric response to the reduction of oxygen, H_2O_2, and nitrite.

1. Introduction

Electron transfer (ET) reactions are crucial in many natural energy conversion processes, from photosynthesis to respiration to the nitrogen cycle, most of which are mediated by proteins. The mechanism of electron transfer has been thoroughly explored in natural proteins.[1] Protein−protein interactions play fundamental roles in controlling the mechanisms of the important biological processes such as gene regulation, enzyme inhibition, and protein self-assembly. The direct electron-transfer reactions can be used to probe the nature of these interactions when the proteins have redox active interactions;[2] these interactions were often investigated by using electrochemical techniques. Usually, the protein electroactive center embeds deeply in the protein structure. Unfavorable orientation of protein molecules on the electrode surface may block the electron transfer between electrode and protein electroactive centers. Moreover, the adsorption of protein molecules onto the bare electrode surface would lead to their denaturation, which also decreases direct electron-transfer rate.[3] Recently, a few methods were proposed to realize the direct electron transfer by the interaction of protein with the functional nanomaterials, polymer films, surfactants, and lipid on the electrode surface.[4] However, there are nearly no reports of using the interaction of proteins to research the direct electron transfer at the electrodes.[5] In this paper, direct electrochemical and electrocatalytic behavior of myoglobin (Mb) immobilized on glass carbon electrode (GCE) by gelatine films was investigated for the first time.

Myoglobin (Mb) is a heme protein in muscle tissue with the functions to store and transport oxygen. The Mb with an α-helical polypeptide chain folds into several segments that serve to stabilize the conformation of iron heme through hydrophobic interaction and hydrogen bonding. Mb is an ideal molecule for the study of electron-transfer reactions of heme proteins because of its commercial availability, a known and documented structure, enzyme-like redox catalytic activity, and numerous studies of its electron-transfer characters have been performed, both homogeneous and heterogeneous.[6] Great efforts have been made to enhance its electron transfer by using mediators, promoters, or some special electrode materials.[7] There are almost no reports about studying the direct electrochemical and electrocatalytic behavior of myoglobin (Mb) with other protein, such as gelatine, on GC electrode.

Gelatine (gel) is a linear polypeptide that consists of different amounts of 18 amino acids. Gelatine was originally selected here because of its excellent membrane-forming ability, good adhesion, biocompatibility, and nontoxicity,[8] which is important for preparing the biosensor and functional devices. Interestingly, the gelatine cannot only be used for immobilization of Mb efficiently, but it also can apparently be used to enhance the direct electron-transfer interaction between Mb and electrode. To gain further information about the structure−function relationship between Mb, gelatine and electrode, we have employed an electrochemical method, circular dichroism (CD), and SDS−PAGE techniques. The results show conclusively that β-type secondary structures in the gelatine can facilitate electron tunneling between Mb and electrode. In additional, Mb shows fine reversible redox process on gelatine modified GC electrodes and keeps its catalytic activity to oxygen, H_2O_2 and nitrite.

2. Experimental Section

2.1. Chemicals. Horse heart myoglobin (Mb, MW, 17 800) from Sigma was used as received without further purification. Gelatine was purchased from the Shanghai Reagent Co. The 0.1 M phosphate buffers of various pHs were prepared by mixing the stock solutions of Na_2HPO_4 and NaH_2PO_4 and adjusted by 0.1 mM NaOH and 0.1 mM H_3PO_4 solutions. Gelatine solution (1%) was prepared by dissolved gelatine flake in warm doubly distilled water. All solutions were made up with redistilled water. All other chemicals were of analytical grade and used without further purification.

2.2. Procedures. Glassy carbon electrodes (GCE, 3 mm diameter) were polished successively with 1.0, 0.3, and 0.05 μm alumina powder on silk, and rinsed thoroughly with doubly distilled water between each polishing step. Next, the polished

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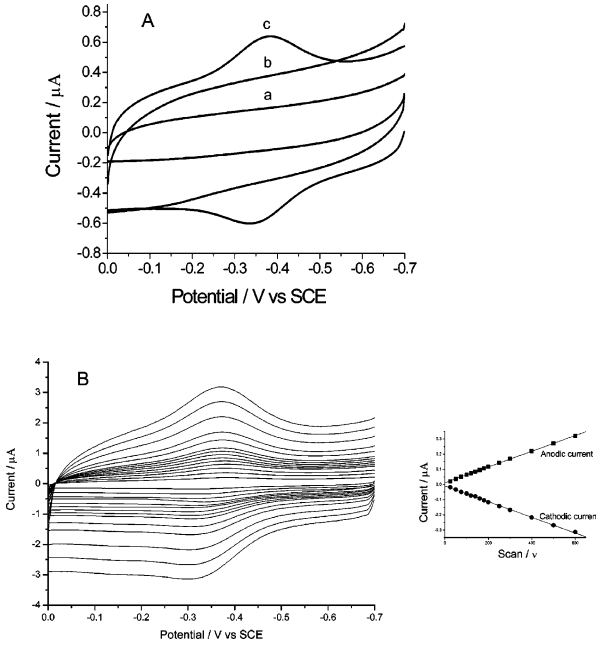

Figure 1. (A) Cyclic voltammograms of bare GC electrode (a), gel/GC electrode (b), and Mb−Gel/GC electrode (c) in pH 7.0 PBS at scan rate of 100 mV/s; (B) Typical cyclic voltammograms of Mb/GCE at different scan rates (from inner to outer): 25, 50, 75, 100, 120, 140, 160, 180, 200, 250, 300, 400, 500, 600 mV s^{-1} in the 0.1 M phosphate buffer (pH 7.0). Inserted figure shows the peak current dependence on the scan rate, υ.

electrode was sonicated in 1:1 nitric acid, acetone, and doubly distilled water, and then allowed to dry at room temperature.

0.1 mg Mb was dissolved in 0.1 mL of gelatine solution (1%), and then 3 μL of formaldehyde solution was added into the enzyme solution. The mixture was hand-mixed completely. The GCE was coated with a drop of 10 μL the resulting mixture, and then was left for at least 24 h at 4 °C. Thus, Mb/gelatine electrodes were obtained. The modified electrode was stored at 4 °C in a refrigerator when not in use.

2.3. Apparatus and Measurements. All electrochemical experiments were carried out in a three-electrode cell controlled by CHI 660 electrochemical workstation (CH Instruments, U.S.A.). The working electrode was a Mb/gelatine modified GC electrode. Reference and counter electrodes were a SCE and platinum wire, respectively.

Prior to the electrochemical measurements, buffer solutions were purified in nitrogen for at least 15 min, and then the nitrogen was bubbled gently through the solutions to exclude oxygen throughout the experiments.

Circular dichroism (CD) spectra were made on a JASCO J-810 from 190 to 330 nm with a 1 nm step resolution, a 1 nm bandwidth. Quartz cuvettes with a 10 mm path length were used.

SDS/gel electrophoresis was performed as described,[22] with 11.5% (w/v) polyacrylamide running gel. Protein samples for SDS/gel electrophoresis were previously heated at 100 °C for 5 min in the presence of 2% (w/v) SDS and 1 mM mercaptoethanol.

3. Results and Discussion

3.1. Direct Electrochemical of Mb Immobilized on the Mb/Gel/GC Electrode. A series of cyclic voltammograms (CV) of gelatine and the Mb/gel modified glassy carbon (GC) electrodes in 0.1 M PBS (pH 7.0) are shown in Figure 1A. Neither the bare glassy carbon electrode nor the gelatine-modified electrode showed any electrochemical response in the phosphate buffer

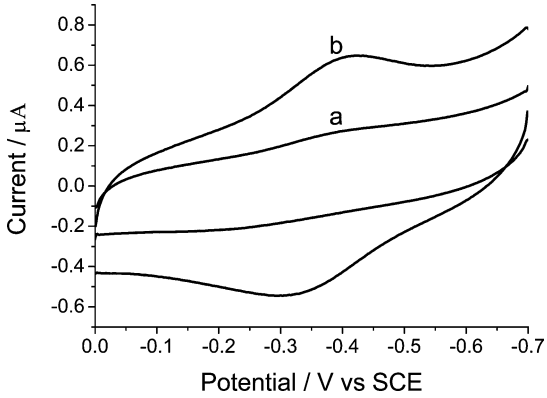

Figure 2. Cyclic voltammograms of bare GCE in 5 mg/mL Mb (a) and in 5 mg/mL Mb +0.2 mL 1% gelatine (b) pH 7.0 PBS at scan rate of 100 mV/s.

(curves a and b in Figure 1A), whereas cyclic voltammogram displayed a pair of well-defined and nearly reversible redox peaks (curve c in Figure 1A) when the GC electrode covered with a monolayer of Mb/gel film. The formal potential (E_0'), taken by the average value of the anodic and cathodic peak potentials, is −370mV (versus SCE.). These results coincide well with heme Fe(III)/Fe(II) couples of the proteins.[9] The CV peak separation (ΔE_p) is 50mV, indicating a fast electron-transfer process.

With an increasing scan rate ranging from 25 to 600 mV s^{-1}, the anodic peak current and the cathodic peak current were proportional to υ (Figure 1B), suggesting a typical surface-controlled electrode process. Although a small peak separation ($\Delta E_p = E_{pa} - E_{pc}$) is observed at low scan rates, the increasing peak separation occurs from the 1 V/s. The electron-transfer rate constant was calculated is about 60 s^{-1}, according to the Laviron's method,[10] which is similar to previous results.[11] It also indicates reasonable fast electron transfers between the immobilized Mb and GC electrode. In this system, the surface concentration of the electroactive proteins (Γ^*) can be estimated by integration of CV reduction peak and applying the formula of $Q = nFA\Gamma^*$, where Q is the integration charge (C) of reduction peak and the charge values (Q) is nearly constant in different scan rates, A is the geometric area of GC electrode (0.07 cm^2) and n and F have their usual meanings. In the range of scan rate from 0.025 to 2 V s^{-1}, the average Γ^* value was 4.3×10^{-11} mol cm^{-2} for the Mb/gelatine film. The percent of the electroactive proteins is about 12.3%. This value is higher than that reported in Nafion−Mb−PC film on PG electrode.[21]

To investigate the effect of gelatine in electron-transfer process, the electrochemical experiments were carried out in 0.1 M PBS with 5×10^{-3} M Mb at the bare GC electrode, the pair of peaks could hardly be observed (Figure 2a). However, when gelatine was added into the solutions, a pair of quasi-reversible peaks could be observed (Figure 2b) clearly. Although we do not know the effect of gelatine on the direct electron transfer of Mb, the different electrochemical behaviors of Mb in the presence and absence of gelatine suggested that gelatine played an important role in facilitating the electron exchange between Mb and GC electrode. We suppose that there might be electron tunneling in Mb and gelatine to promote the electron-transfer reaction between the Mb and electrode.

3.2. Spectroscopic Characterization. The protein environment is composed of various arrangements of secondary structures such as α-helixes, β strands, and random coils, as well as hydrogen bond and Vander Waals contacts, surrounded by lipids or water molecules. It was reported that the secondary

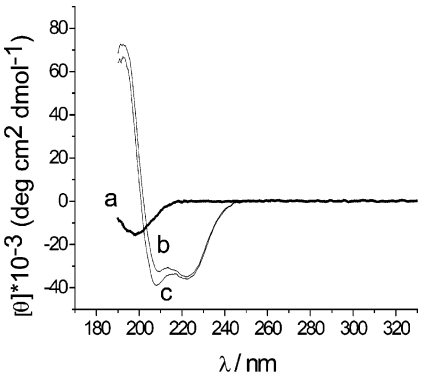

Figure 3. UV circular dichroism spectra (190−330 nm) of gelatine solution (a); Mb solution (b); the mixture solution of Mb and gelatine with formaldehyde cross-linking (c).

structure played an important role in electron tunneling in proteins.[12] Therefore, determining which part of the protein environment is most significantly used in the electron tunneling between Mb and gelatine is an intriguing. Circular dichroism (CD) has commonly been employed to study secondary structural information on peptides and proteins.[13] Figure 3 shows the UV circular dichroism (CD) spectra for gel, native Mb and Mb/gel cross-linked with the formaldehyde. The curve a in Figure 3 of the negative bands at 197 and 216 nm indicates that the secondary structures of gel include β turn and random coil. The two typical negative extrema at 208 and 222 nm (curve b in Figure 3), which would be related to the α-helix structure, was observed in the native CD spectra of myoglobin, indicating that the proteins would be α-helix-rich. It was reported that the electronic coupling in structure could produce when β-sheet proteins exist, and the β-sheet are tightly knit structures that efficiently and isotropically mediate distant electronic couplings.[14] It was also reported that the β-turn secondary structure could change to β-sheet in gelatine.[15] Therefore, we can logically explain the electrochemical experimental results in view of the secondary structure of Mb and gelatine. The schematic view is shown in Figure 4. The direct electron transfer of Mb without the gelatine is difficult in the electrode due to the high anisotropic of α-helical. When gelatine is present, the gelatine can couple with the Mb molecules, the electron transfer of gelatine is isotropically, the gelatine can act as an electron tunneling between the Mb and electrode, so the direct electrochemical response of Mb is very obvious.

Because the gelatine has certain solubility in water, the gelatine molecules can be cross-linked by the formaldehyde and some new chemical bonds can be created. The gelatine can form a three dimensional net structure to immobilize Mb molecules on electrode surface to get an electrochemical biosensor. After the Mb/gel solution is cross-linked with formaldehyde, the CD curve c, shown in Figure 3, indicates that the secondary structure of Mb has no changes. The results also prove that the Mb bioactivity has no changes and there is a good biocompatibility between the gel and Mb.

3.3. SDS−PAGE Characterization. Herein, the Mb/gel solution must be cross-linked with formaldehyde to keep the biosensor stable. Formaldehyde, the simplest and tight (2 Å) cross linking agents, has the broadest reaction specificity. In addition to amine group of lysine, it reacts with the side chains of cysteine, tyrosine, histidine, tryptophan, and arginine. Although formaldehyde contains a single functional group, it can react bifunctionally, and therefore, cross-linked gelatine to obtain network film for entrapment enzymes or proteins.[16] For Mb, α-helix structure, the covalent pathway could be quite long compared to the through-space distance, so the electron may compromise by tunneling gelatine through a combination of covalent bonds or noncovalent hydrogen bonds interactions.[17] To research the combination, the SDS−PAGE experiments were carried out for different samples, and the results were shown in Figure 5. Lanes 1 and 2 correspond to the gel and Mb, lane 3 corresponds to the mixture of gel and Mb, and lane 4 corresponds to the gel−Mb mixture with formaldehyde cross-linking. Comparing lane 3 with lane 4, no more molecules with the big molecular weight are observed in lane 4. After adding the formaldehyde, there are three possible reactions in the solution. The first is the reaction with gel to each other; the second is the cross-linking between the gel and Mb, the third is the cross-linking with Mb molecules to each other. Because the more active points presented on the chain, the gel should be easier to cross-link to each other. Comparing lanes 2 and 3 with lane 4, the band of Mb molecules hardly changes. Therefore, the Mb molecules cross-linked each other was very little. The results proved that the formaldehyde could be used to cross-link the gel molecules. The combination of electron tunneling in Mb and gel is noncovalent hydrogen bonds.[18]

The glutaraldehyde was selected as cross-linking reagent to increase the covalent link between the Mb and gelatine in lane 5 of Figure 5. The bands of lane 5 are different from those of lane 2, 3, and 4, suggesting covalent link forming between the Mb and gelatine. The electrochemical experimental results showed that the direct electron-transfer response was weaker at this condition than that at formaldehyde as a cross-linking reagent. The curves of the CV are shown in Figure 6. The results clearly indicate that the hydrogen bonds could transfer electrons more efficiently than the covalent bonds between the Mb and gelatine.

3.4. Effects of Solution pH on Direct Electron Transfer of Mb/Gel Modified GCE. The direct electrochemistry of Mb immobilized on GCE by incorporated with gelatine film showed a dependence on solution pH (Figure 7). An increase of solution pH from pH 4 to 8.3 led to a negative shift of both reduction and oxidation peak potentials. The slope value of formal potential was −51 mV/pH (inset in Figure 7), which is close to the reported value of −55.7 mV/pH and −52 mV/pH,[19]

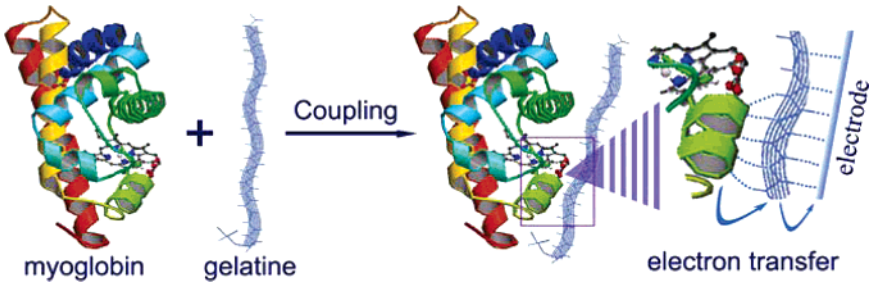

Figure 4. Schematic view of the interaction between the Mb and gelatine and the electron transfer between the Mb and electrode.

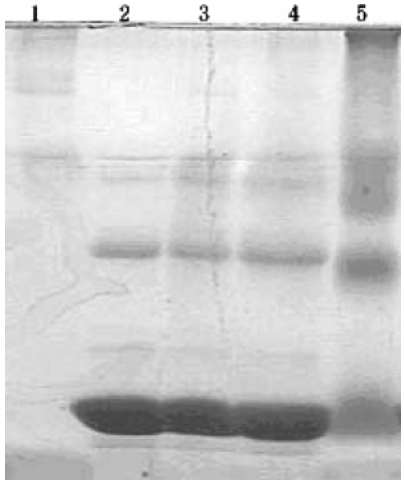

Figure 5. SDS−PAGE analytical graphs of different samples. Lane 1 is gelatine; Lane 2 is the Mb; Lane 3 contains the Mb and gelatine; Lane 4 shows the mixture of Mb and gelatine cross-linked by formaldehyde; Lane 5 shows the mixture of Mb and gelatine cross-linked by glutaraldehyde.

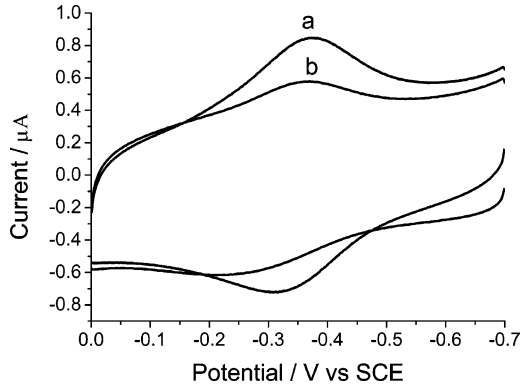

Figure 6. Cyclic voltammograms of Mb/gelatine cross-linked with the (a) formaldehyde and (b) glutaraldehyde film modified GCE in 0.1 M PBS (pH 7.0) at scan rate of 100 mV/s.

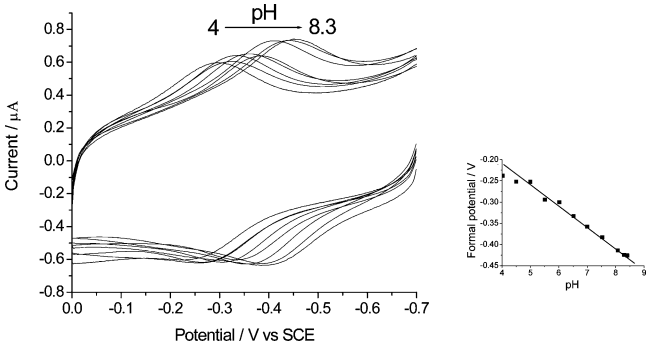

Figure 7. Cyclic voltammograms of Mb−gel/GCE in 0.1 M PBS with various pH values at scan rate of 100mVs^{-1}. Inset figure shows effect of pH on formal potential.

respectively. The results indicate that a single protonation accompanies the electron transfer of Mb Fe(III) to electrodes and the catalytic process of electron transfer is a nearly reversible process. And the results can also indicate that gelatine film can provide a suitable microenvironment for Mb.

3.5. Catalytic Reactivity. Electrocatalytic activity of Mb/gel film modified GC electrode toward various substrates of biological or environmental significance, such as hydrogen peroxide, oxygen, and nitrite, was examined and characterized by CV. The typical catalytic reduction peak of the H_2O_2 is observed at −0.37 V in Figure 8. With the increasing of the

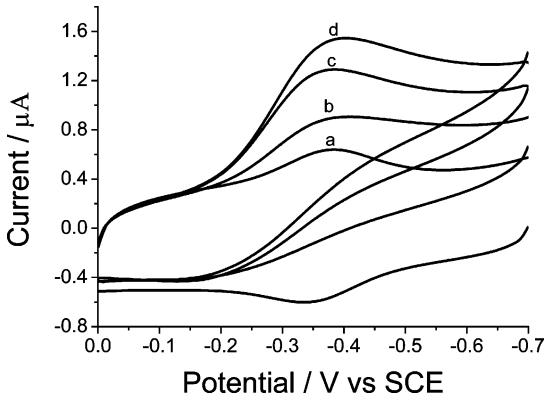

Figure 8. Cyclic voltammograms of Mb−gelatine−GC in the absence of H_2O_2 (a), and in the presence of 0.3 mM (b), 0.6 mM(c), 0.9 mM H_2O_2 (d) in pH 7.0 PBS at scan rate of 100 mV/s.

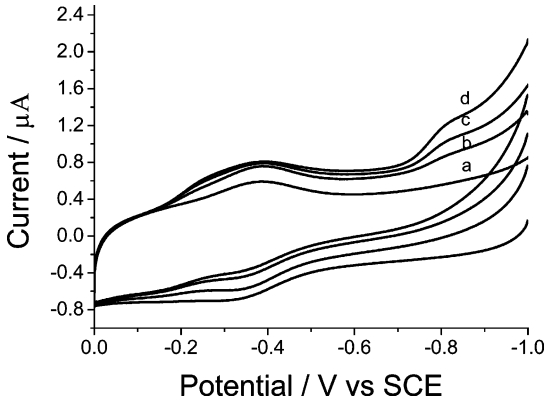

Figure 9. Cyclic voltammograms of Mb/gelatine film modified GCE in a 0.1 M PBS (PH 7.0): (a) before and after the addition of (b) 4 mM, (c) 16 mM, and (d) 32 mM $NaNO_2$. Scan rate: 100 mV/s.

concentration of the H_2O_2, the reduction current is increasing. The results indicate that the Mb in the modified electrode can partially react with H_2O_2 in the electrochemical process. The mechanism of catalytic reduction of hydrogen peroxide at Mb/gel film modified GC electrode can be elucidated by the pathway suggested by Rusling.[20] When the direct electrochemical reduction of the Fe(III)Mb to Fe(II)Mb is in the electrode, the Fe(II)Mb can be oxidized with H_2O_2 to transfer to the Fe(III)Mb quickly. The reaction can increase the reduction current to form the catalytic current. The similar catalytic results were observed near O_2 in the same experiments on Mb/gel/GC electrodes (not shown).

Catalytic reduction of nitrite was also studied at Mb/gel/GC electrodes and the result is shown in Figure 9. With the addition of NO_2^- in a pH 7.0 buffer, a new reduction peak at about −0.8V is observed, and the further addition of NO_2^- causes an increase of the peak current.

These results indicated that Mb keeps its bioelectrocatalytic activity after adsorption on the surface of the gelatine modified GC electrode.

4. Conclusion

In summary, the reported results demonstrated that quasi-reversible, surface-controlled electron-transfer kinetics for Mb was observed by the interaction between gelatine and Mb molecules. The β-type chain in gelatine can interact with α-helical chain to form an electron tunneling to promote the protein direct electrochemistry, and it had been proved that the hydrogen bonds have more coupling efficiency than the covalent bonds for electron transfer. This mechanism is very significant

for studying the interactions between proteins and understanding many biological processes.

Moreover, Mb can retain its near-native secondary structure in gelatine films, and exhibit a rapid amperometric response to the reduction of oxygen, H_2O_2, and nitrite. This new type of protein−polypeptide films might have potential applicability in constructing biosensors or bioreactors based on the direct electrochemistry of enzyme without using any mediator.

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