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# Stabilization Role of a Phenothiazine Derivative on the Electrocatalytic Oxidation of Hydrogen via *Aquifex aeolicus* Hydrogenase at Graphite Membrane Electrodes

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The [NiFe] membrane-bound hydrogenase from the microaerophilic, hyperthermophilic *Aquifex aeolicus* bacterium (*Aa* Hase) presents oxygen, carbon monoxide, and temperature resistances. Since it oxidizes hydrogen with high turnover, this enzyme is thus of particular interest for biotechnological applications, such as biofuel cells. Efficient immobilization of the enzyme onto electrodes is however a mandatory step. To gain further insight into the parameters governing the interfacial electron process, cyclic voltammetry was performed combining the use of a phenothiazine dye with a membrane electrode design where the enzyme is entrapped in a thin layer. In the absence of the phenothiazine dye, direct electron transfer (DET) for $H_2$ oxidation is observed due to *Aa* Hase adsorbed onto the PG electrode. An unexpected loss of the catalytic current with time is however observed. The effect of toluidine blue O (TBO) on the catalytic process is first studied with TBO in solution. In addition to the expected mediated electron transfer process (MET), TBO is demonstrated to reconnect directly some *Aa* Hase molecules possibly released from the electrode but still entrapped in the thin layer. On adsorbed TBO the two same processes occur demonstrating the ability of the TBO film to connect *Aa* Hase via a DET process. Loss of activity is however observed due to the poor stability of adsorbed TBO at high temperatures. *Aa* Hase immobilization is then studied on electropolymerized TBO (pTBO). The effect of film thickness, temperature, presence of inhibitors and pH is evaluated. Given a film thickness less than 20 nm, $H_2$ oxidation proceeds via a mixed DET/MET process through the pTBO film. A high and very stable $H_2$ oxidation activity is reached, showing the potential applicability of the bioelectrode for biotechnologies. Finally, the multifunctional roles of TBO-based matrix are underlined, including redox mediator, *Aa* Hase anchor, but also buffering and ROS scavenger capabilities to drive pH local changes and avoid oxidative damage.

## 1. Introduction

In a future "green" hydrogen economy, the use of biocatalysts such as hydrogenases, the enzyme that catalyzes the hydrogen conversion according to $H_2 \leftrightarrows 2H^+ + 2e^-$, emerges as a replacement of chemical catalysts in fuel cells.[1,2] Great improvements in the cell design and the environmental impact are expected thanks to the properties of the biocatalyst. The main advantage of such biofuel cells is the availability and biodegradability of the enzyme, coupled with power densities comparable to platinum catalysts at least in some hydrogen pressure conditions.[3,4] In addition, the high specificity toward $H_2$ can eliminate the need for a membrane separator. Two major bottlenecks have to be overcome, however, before the development of hydrogenase-based biofuel cells: most hydrogenases are oxygen sensitive and have limited lifetimes especially once immobilized onto an electrode surface. Recently, genetic manipulation allowed the $O_2$ sensitive [NiFe] hydrogenase from *Desulfovibrio fructosovorans* to be converted into a resistant enzyme.[5] This is however a unique example in the literature, and catalytic efficiency was proved for only several minutes. Another very attractive way is to work with naturally resistant hydrogenases. This can be achieved via the use of hydrogenases from aerobic bacteria such as *Ralstonia eutropha*.[6] Furthermore, during the past decade, a large number of "exotic" microbial species have been discovered. These organisms are a source of stable enzymes at extreme temperatures, pressures, or pH. In most of them, hydrogenases have been detected. Among these organisms, *Aquifex aeolicus* (*Aa*) is a hydrogen-oxidizing, microaerophilic bacterium growing at 85 °C.[7] A membrane-bound [NiFe] hydrogenase (*Aa* Hase) has been identified in *Aa* that is involved in a complex with a diheme cytochrome *b* anchored in the membrane.[8] Previous studies have shown that *Aa* Hase is rather stable at elevated temperatures and exhibits tolerance to oxygen and CO that makes it a good candidate for biotechnological uses.[9,10]

Various strategies have been developed, essentially applied to mesophilic hydrogenases, in view of electrode architecture able to provide efficient hydrogenase immobilization. The widely used procedure is based on simple adsorption of the enzyme onto PG electrode. In this case, given an active PG edge surface, direct interfacial electron transfer is achieved, with no need of any redox mediators. Over the past decade this concept has provided

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important new insights on electron and proton transfer mechanisms during $H_2$ oxidation by "classical" mesophilic hydrogenase.[11−13] More recently, adsorption of *Aa* Hase onto electrodes was shown to provide an efficient direct interfacial electron transfer for $H_2$ oxidation.[9,10] In contrast to [NiFe] Hase from *Desulfovibrio fructosovorans*, it was however demonstrated that *Aa* Hase was adsorbed according to various orientations. Two populations were shown to exist at the electrode interface. The first one presented its distal FeS cluster (the surface cluster which is the entry/exit point of the electrons) at a tunnel distance from the electrode surface so that direct electron transfer (DET) could be achieved. In the second one the FeS distal cluster was too far from the electrode so that $H_2$ oxidation could occur only via the aid of a redox mediator. The addition of a phenothiazine dye in solution as a redox mediator was thus shown to increase hydrogen oxidation current.

Simple enzyme adsorption is however not convenient for long-term efficiency. Attempts to firmly attach hydrogenase to electrode interfaces while enhancing catalytic efficiency have used various approaches: biocomposite electrode based on clay layers[14] or carbon nanotube networks,[9,13,15,16] thiol-functionalized gold electrodes,[9,17] and finally a novel concept based on the reconstitution of the electron transfer chain into liposomes.[18] The entrapment of mesophilic hydrogenase within viologen-containing films was the only example in the literature of efficient and promising catalysis combining multilayered or electropolymerized films and mediator coimmobilisation.[3,14,19]

Nevertheless, although the immobilization process has been reported to stabilize the hydrogenase during storage,[15,20] a continuous decrease of the catalytic current was most often pointed out along with $H_2$ electrocatalytic oxidation. This decrease was much more pronounced at elevated temperature.[9,21] No discrimination between release of the enzyme from the interface, reorientation of the enzyme, or denaturation was possible at that time. An exception was reported based on encapsulation of *Aa* Hase in a single-walled carbon nanotube (SWCN) network.[9] In this latter case, it was supposed that favorable hydrophobic interactions in the SWCN cavities served to stabilize the enzyme. Moreover, a recent surface-enhanced IR absorption study performed on a mesophilic hydrogenase attached to thiol-modified gold electrode concluded that the current decrease with time did not result from hydrogenase leaching but was related to slow degradation of the active site, more probably by residual traces of $O_2$.[17]

The membrane electrode offers one possibility to revisit this process. In this configuration the enzyme is imprisoned in a thin layer between the electrode surface and a dialysis membrane, while the substrate and the redox mediator are able to diffuse through. In this work we propose a new strategy for *Aa* Hase immobilization in order to study and prevent the loss of catalytic activity with time. This is based on the combined use of a phenothiazine dye, toluidine blue O (TBO), with the membrane electrode design. $H_2$ oxidation by *Aa* Hase at elevated temperatures is thus explored at the membrane electrode with the phenothiazine used under three different modes, in solution, adsorbed onto the electrode, or electropolymerized. The analysis of DET versus mediated electron transfer (MET) currents allows discriminating between leaching from the electrode or irreversible degradation of the enzyme. Furthermore, thanks to the ability of TBO to form conductive electropolymerized films, the advantages of coimmobilization of *Aa* Hase with the redox mediator in terms of stability and efficiency of $H_2$ oxidation process are discussed. This design is shown to be suitable for biotechnological applications.

## 2. Experimental Section

**2.1. Reagents and Instruments.** *Aquifex aeolicus* membrane-bound hydrogenase (*Aa* Hase) was purified as described by Luo et al.,[9] except that Amicon PM 100 was used for the concentration step. Toluidine blue O (3-amino-7-dimethylamino-2-methyl-phenothiazinium chloride (TBO)) and hexacyanoferrate(III) ($Fe(CN)_6^{3-}$) were purchased from Sigma and Fluka, respectively. They were used without further purification. All other chemicals were reagent grade. Cytochrome $c_3$ from *Desulfovibrio gigas* (Dg) was prepared and purified as previously described.[22] Carboxylic acid functionalized single-walled carbon nanotubes (SWCN-$CO_2H$) from Sigma were used as received. According to the supplier, the SWCN-$CO_2H$ sample has an average internal diameter of 4.5 nm and an average length of 1 $\mu$m. Stable dispersions of nanotubes were performed with the aid of sonication for 30 min. The carbon nanotube concentration in the dispersions was 1.3 mg/mL in water.

All solutions were prepared with distilled, deionized water. 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) was used for hydrogenase dilution and as the supporting electrolyte at pH 7.2 and 50 mM concentration. Other buffers were used for TBO electropolymerization experiments: 25 mM Tris-HCl pH 9.5 and Tris-acetate pH 4.7.

Cyclic voltammetry (CV) was done using a PARSTAT 2273 potentiostat from Princeton Applied Research (PAR). A conventional three-electrode system was used with a Ag/AgCl/NaCl (sat.) reference electrode and a gold wire as auxiliary electrode. All potentials were quoted against the Ag/AgCl reference. Prior to each experiment, the solutions were deoxygenated by bubbling with high-purity nitrogen or hydrogen. The temperature of the electrochemical cell was regulated using a water bath. The reference electrode was separated from the warmed electrolyte using a side junction maintained at room temperature.

**2.2. Design of the Working Electrode.** The working electrode was constructed from 3 mm rods of pyrolytic graphite (PG) inserted in Peek polymer casings from Bio-Logic SAS. Usually, the PG electrode was carefully polished on wet fine emery papers (PRESI, P800 then P1200). After polishing, the PG electrode was washed thoroughly with water, followed by sonication and rinsed again with water.

Unless specified, the membrane electrode technology was used for electrochemical measurements. Briefly, an aliquot of 1 $\mu$L of the buffer containing or not the enzyme was deposited on the freshly polished surface of the electrode. The electrode was then gently pressed against a square piece of dialysis membrane (Visking membrane − MWCO 6000−8000). A rubber ring was fitted around the electrode body so that the volume between the membrane and the electrode surface corresponded to a uniform thin layer.[23] Then, the thus-mounted membrane electrode was placed in the electrochemical cell containing the solution to be studied.

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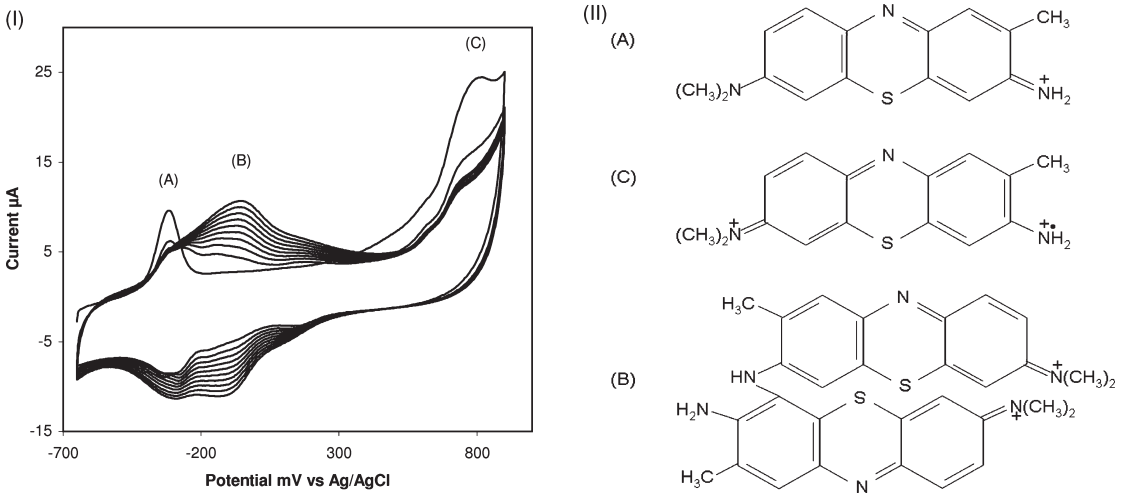
**Scheme 1. Electropolymerization Process of TBO**[a]




[a] (I) CVs obtained at the PG electrode in 25 mM Tris-HCl buffer pH 9.5 containing 160 $\mu$M TBO, 50 mV $s^{-1}$; (II) proposed TBO species associated with redox signals in (I).

**2.3. Adsorption of TBO.** The adsorption of TBO was performed by immersion of the graphite electrode into a 160 $\mu$M aqueous TBO solution for 1 min. The electrode was then carefully rinsed with water.

**2.4. Electropolymerization of TBO.** The polymerization of TBO at the graphite electrode was carried out by cyclic potential sweep from −0.65 to 0.9 V at a scan rate of 50 $mVs^{-1}$ in 160 $\mu$M TBO solution. The thus-modified PG electrode was rinsed with water for further use.

TBO electropolymerization to form poly(toluidine blue O) film (pTBO) is well documented.[24−27] Briefly, as shown in Scheme 1, cycling the PG electrode in 160 $\mu$M TBO solution from −0.65 up to +0.9 V results in the appearance of new redox waves (B) in addition to redox peaks for TBO monomer (A). Peaks B are linked to the appearance of process C. This process is due to the oxidation of the amino group of TBO to form a cation radical, initializing the electropolymerization *via* carbon−nitrogen coupling reactions to form pTBO. Peaks B which continuously increase with increasing number of cycles, are ascribed to the redox couple of the polymer. The number of cycles controlled the thickness of the film. Electropolymerization was performed in 25 mM Tris-HCl pH 9.5, unless otherwise specified. The electropolymerized PG electrode was then carefully rinsed three times with water before further use.

## 3. Results and Discussion

**3.1. Direct Hydrogen Oxidation with *Aa* Hase Entrapped in a Thin Layer Configuration.** In the previous work devoted to the electrochemistry of *Aa* Hase, it was shown that enzyme adsorption at the PG electrode followed two concomitant steps. In a first step, the enzyme in solution progressively adsorbed onto the electrode surface. This was reflected by the increase of the catalytic current for $H_2$ oxidation. Once adsorbed, a decrease with time in the direct catalytic current for $H_2$ oxidation was however recorded.[9] At the PG membrane electrode filled with 1 $\mu$L of 1 $\mu$M *Aa* Hase an $H_2$ dependent oxidation current occurs in the absence of any redox mediators (Figure 1). The current−potential response on the first potential scan is very similar to that previously obtained

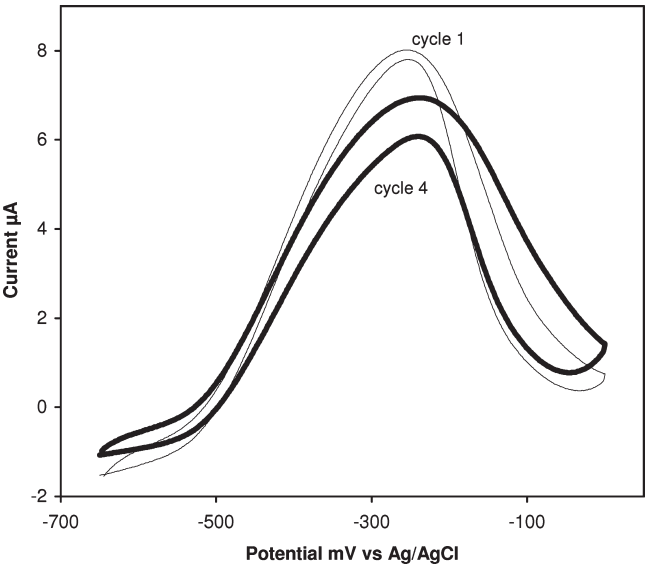



**Figure 1.** Catalytic oxidation of hydrogen with *Aa* Hase entrapped in a thin layer at the membrane PG electrode as a function of the CV cycle number: (fine line) first cycle, (bold line) fourth cycle; 50 mM HEPES buffer pH 7.2, 65 °C, $H_2$ atm, 5 mV $s^{-1}$.

with *Aa* Hase adsorbed at the PG electrode with no membrane,[9] or to preadsorbed *Aa* Hase at the membrane PG electrode. It is thus related to the oxidation of $H_2$ by the adsorbed *Aa* Hase layer that is directly connected at the electrode interface through the distal FeS cluster.

Briefly, the catalytic $H_2$ oxidation process starts when the potential is raised around 100 mV above the formal $H^+/H_2$ potential value at pH 7.2. The reversible inactivation process resulting in the peak like response occurs at potentials above −250 mV. Thus the main kinetic data shared by $O_2$ insensitive hydrogenases, i.e., *Ralstonia eutropha* [NiFe] membrane−bound hydrogenase and *Aa* Hase, are preserved at the membrane electrode. It can be concluded that the membrane configuration does not significantly modify the interaction between the enzyme and the electrode surface. However, from Figure 1 it is clear that the entrapment of *Aa* Hase within the membrane layer does not prevent the loss in the catalytic current. A 15% decrease in the catalytic current is already recorded after

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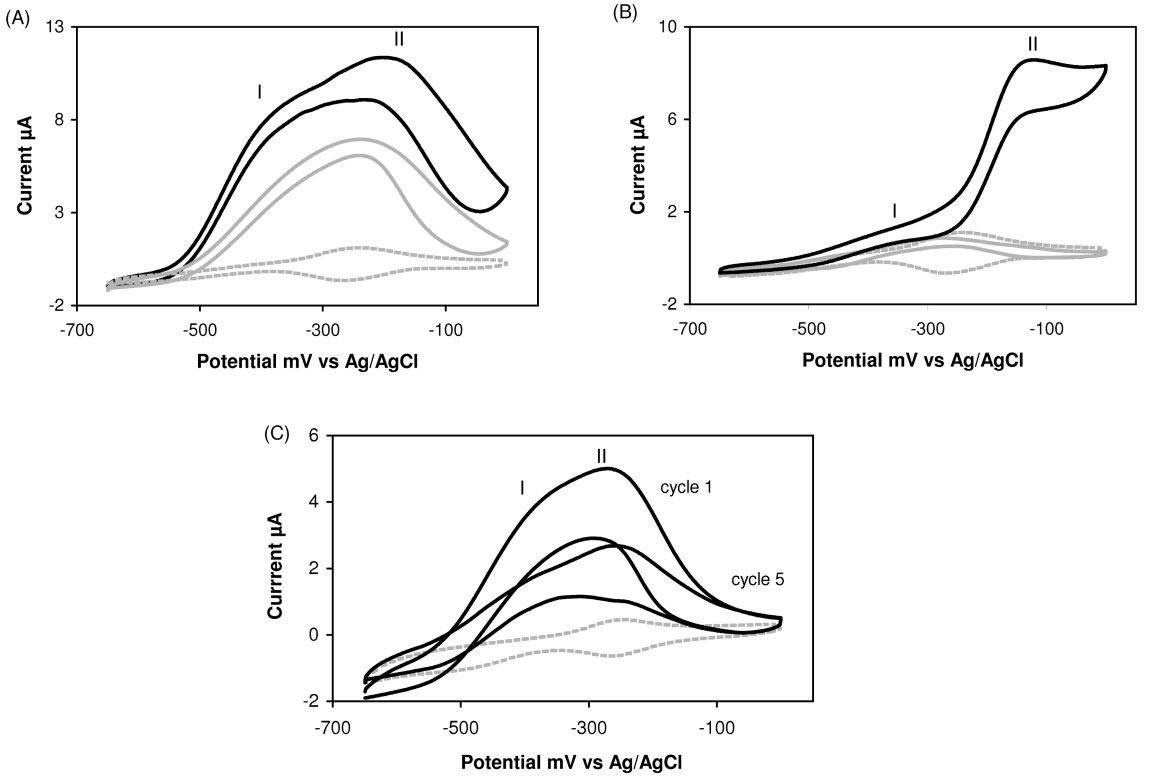


**Figure 2.** Direct (process I) versus TBO mediated (process II) electron transfer for $H_2$ oxidation via *Aa* Hase in a thin layer at the membrane PG electrode: in the presence of 16 $\mu$M TBO in buffer solution (A) at the emery polished PG electrode and (B) at the alumina polished PG electrode; (C) with adsorbed TBO as a function of the CV cycle number; 50 mM HEPES buffer pH 7.2, 65 °C, $H_2$ atm., 5 mV $s^{-1}$. Grey lines correspond to $H_2$ oxidation via *Aa* Hase in a thin layer at the membrane PG electrode in the absence of TBO. Dotted lines correspond to the CV signals for TBO alone at the PG membrane electrode.

only four continuous cycles. Actually the thin layer was evaluated to be around 10 $\mu$m thick.[23] It is thus understandable that the retention of *Aa* Hase in the thin layer is not a sufficient condition to ensure the DET process. However, we expected that equilibrium could have been reached in the adsorption−desorption process, if occurring, with a steady-state catalytic current. But no stable current has been observed at least for 3 h cycling. From these experiments and in agreement with the reported spectroscopic data,[17] it is demonstrated that other factors than simple release are implied in the decrease of the catalytic current with time.

**3.2. Direct versus Mediated Electron Transfer Using Toluidine Blue O Redox Mediator.** The oxidation of $H_2$ by *Aa* Hase entrapped in the thin layer configuration was further explored by using TBO as a redox mediator. It was expected that the *Aa* Hase molecules supposedly released and/or reoriented at the interface could be connected via the redox mediator. TBO's redox potential is adapted to *Aa* Hase-based reaction. TBO has been furthermore demonstrated to play a beneficial role (either as a monomer or in its electropolymerized form) in various biosensor recent applications.[24,28−32]

*Addition of TBO in Solution*. Figure 2A,B compares the CV curves obtained under $H_2$ atmosphere at 65 °C with 1 $\mu$L of 1 $\mu$M *Aa* Hase at the PG membrane electrode before and after TBO addition in solution. For these experiments, continuous cyclic voltammetry was performed at the *Aa* Hase membrane PG electrode. Before TBO addition, as described in the previous section, the catalytic current decreased within the cycles. At the end of the fifth cycle, 16 $\mu$M TBO was added in the solution. Two main features can be drawn from the comparative CV responses (Figure 2A). First, the DET process potential (process I) is negatively shifted up to 50 mV. This point will be discussed in the following paragraph. A simultaneous increase in the DET current is observed up to 30%. In other words, the system works such that some disconnected and/or new *Aa* Hases are available to exchange directly with the electrode interface. Second, a new oxidation process (process II) appears at the oxidation potential for TBO, related to $H_2$ oxidation mediated by TBO. However, above all a high stability of the electrochemical signal in the presence of TBO in the solution is recorded. No loss of the catalytic current is observed by cycling in the window −0.65/0 V during 3 h. TBO thus acts not only as the expected redox mediator but also plays a role in the increase and stabilization of the direct catalytic current.

To discriminate the possible roles of TBO in the direct $H_2$ oxidation, the influence of the graphite structure was studied by performing the same experiments but at the alumina-polished PG electrode (Figure 2B). Important features are revealed. At first, the DET process (process I) is much lower than that recorded at the emery-polished PG electrode. The addition of 16 $\mu$M TBO in the solution, allows the DET current to increase as observed with the emery-polished PG electrode. But essentially, it permits to connect many hydrogenases via the redox mediator so that the electron transfer proceeds now in the majority via the MET process. It is known that the mode of polishing affects the hydrophobicity of the surface.[33] However, we previously demonstrated that *Aa* Hase adsorption could efficiently occur both at hydrophobic or

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hydrophilic modified gold electrodes, with no preferential orientation.[9] In order to search for the reason of such a different behavior between emery and alumina polishing, the membrane was pulled off after running the CV curve in Figure 2B, and the PG electrode was transferred into 50 mM HEPES, pH 7.2. The resulting CV curve consisted only in a weak DET process with complete disappearance of the MET process. When TBO was added again in solution, the two DET and MET processes were enhanced describing only a small decrease in the current values (data not shown). From this experiment it must be concluded that TBO do act as an anchor for efficient *Aa* Hase orientation at the electrode. It must also be concluded that the MET current which is favored at the alumina polished-surface originates from adsorbed *Aa* Hase molecules that are oriented with the distal FeS cluster at a tunnel distance from the electrode so that DET cannot be achieved.

As can be seen in Figure 2A,B, the electron transfer process shifts from a major DET to a major MET when working at the emery and alumina-polished PG electrodes, respectively. The calculation of the kinetics constant is still an unresolved problem in the case of hydrogenase-based reactions because the amount of active enzymes which is engaged in each process is unknown. Indeed, the appearance of a non catalytic signal for hydrogenases has been successfully observed in only rare cases.[12] Assuming that one monolayer of adsorbed *Aa* Hase is involved in each process (7 pmol/cm$^2$), the interfacial electron transfer constant, $k_{ET}$, and the intermolecular electron transfer constant, $k_{TBO}$, can be calculated from the limiting DET and MET currents respectively.[34] Values of 160 $s^{-1}$ and $7 \times 10^6$ $M^{-1}s^{-1}$ are obtained for $k_{ET}$ and $k_{TBO}$, respectively. These values are most probably under-evaluated but reflect the high activity of *Aa* Hase adsorbed at the electrode.

*TBO Adsorbed at the Electrode Surface*. It is well-known that phenothiazine dyes readily adsorb onto graphite electrodes.[29] The question thus arises if the increase in available *Aa* Hases and in the stabilization of the electrocatalytic signal for $H_2$ oxidation could be driven by adsorbed TBO monomer. In agreement with previous reports,[29] preadsorbed TBO onto PG electrode gives well-defined cathodic and anodic peaks at $E_m = -255$ mV (Figure 2C). The process is more or less stable at 65 °C, leading to a slow progressive decrease of the redox peaks. When the electrode coated with adsorbed TBO and further modified with *Aa* Hase via the membrane configuration is run at 65 °C under $H_2$, DET and MET processes for $H_2$ oxidation occur (Figure 2C). A first conclusion is that *Aa* Hase is efficient for $H_2$ oxidation through a layer of adsorbed TBO. But notably, the same processes are observed whatever the mode of polishing of the electrode, namely with emery or alumina, in marked contrast with the former results obtained with TBO in solution. Two main roles played by TBO can be drawn from these experiments. First, adsorbed TBO is confirmed as an anchor for *Aa* Hases, thus explaining the increase in the DET process upon TBO addition in the solution, even on alumina-treated PG electrode where *Aa* Hase can be only weakly directly connected. These sites may be related to the aromatic rings or to the positive charges bared by the TBO molecules. Second, adsorbed TBO is still able to mediate $H_2$ enzymatic oxidation. No stable electrocatalytic signal can be however obtained with time. TBO monomer amounts in the film are not sufficient and stable enough to ensure a constant connection of *Aa* Hases.

**3.3. Electroenzymatic Hydrogen Oxidation onto Electropolymerized Toluidine Blue Film.** To enhance the number of available mediator molecules at the electrode interface, electropolymerized TBO films were next constructed. It was expected that a higher and constant amount of *Aa* Hase could be connected by this way for $H_2$ oxidation.

**Table 1. Evolution of pTBO Film Parameters As a Function of the Number of Cycles Used for Electropolymerization**

| cycles | charge amount, $\mu$C cm$^{-2}$ | surface concentration, $10^9$ $\Gamma$ mol cm$^{-2}$ | thickness, nm |
|---|---|---|---|
| 2 | 295 | 1.5 | 8 |
| 5 | 635 | 3.3 | 17 |
| 10 | 920 | 4.8 | 24.5 |
| 20 | 1130 | 5.9 | 30 |
| 40 | 1415 | 7.3 | 38 |

*Stability and Electronic Conductivity of pTBO Films*. The stability of the electropolymerized film was followed as a function of temperature. The pTBO-modified PG electrode was rinsed carefully with water and dipped in 50 mM HEPES buffer pH 7.2. The typical CV curves obtained on a pTBO modified electrode after 20 cycles of polymerization when the temperature is increased in the range 27−60 °C are given in Supporting Information (Figure S1). At 27 °C, two redox processes, respectively at −225 and −25 mV, characterize the CV behavior of the pTBO film. In agreement with previous works[24] the more positive couple is ascribed to the redox behavior of the polymer. The more negative couple is admitted to be due to the TBO monomer strongly adsorbed to the polymer, or a monomer-type conjugation in the polymer. The peak currents of the two redox couples are linearly proportional to the scan rates (data not shown), indicating a surface confined process occurs at the pTBO film electrode. When the temperature is increased, the monomer-like redox current continuously diminishes while the polymer redox current keeps stable. Within three hours at 60 °C, no decrease of the polymer redox signal is observed.

The diffusion of substrates and products are expected to be more efficient at thinner polymer films.[31] Conversely, the efficiency of the catalysis might increase with increasing amount of polymer serving as redox mediator and/or anchoring sites in the matrix. The number of cycles used for generating the polymerized film was thus varied from 2 to 40 cycles. In each case, the amount of charge from the integration of the redox peaks for pTBO obtained after transfer of the modified electrode in 50 mM HEPES, pH 7.2 at 65 °C was calculated. It allows the determination of the surface coverage, then the film thickness, assuming that the unit cell length for pTBO is 1 nm.[35] The values are reported in Table 1. Clearly, the charge, thus the film thickness increases with increasing cycles, taking a value of 24.5 nm after 10 cycles of electropolymerization. The film thickness tends to level off after 10 cycles.

The electronic conductivity of these films was first evaluated using $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ and *Desulfovibrio gigas* (Dg) cytochrome $c_3$. These two probes were chosen because of their negative charge, which does not impede their interaction with the positive pTBO film, and their different sizes from a small redox probe to a large protein. Furthermore, Dg cytochrome $c_3$ is a well characterized protein which behaves as a fast reversible electrochemical system at PG electrodes, exhibiting redox potentials in the range −450/-500 mV. It is observed that $Fe(CN)_6^{3-}$ is electrochemically active in the whole range of pTBO thickness films, with no change of the redox peak currents (data not shown). Conversely, Dg cytochrome $c_3$ electrochemical signal is only observed for the thinnest film, ie around 15 nm. In this case, the intensity of the signal is not changed compared to the one obtained at the bare PG electrode (Supporting Information, Figure S1), indicating that weak changes in the electroactive surface occurred. Dg cytochrome $c_3$ is a 12 kDa

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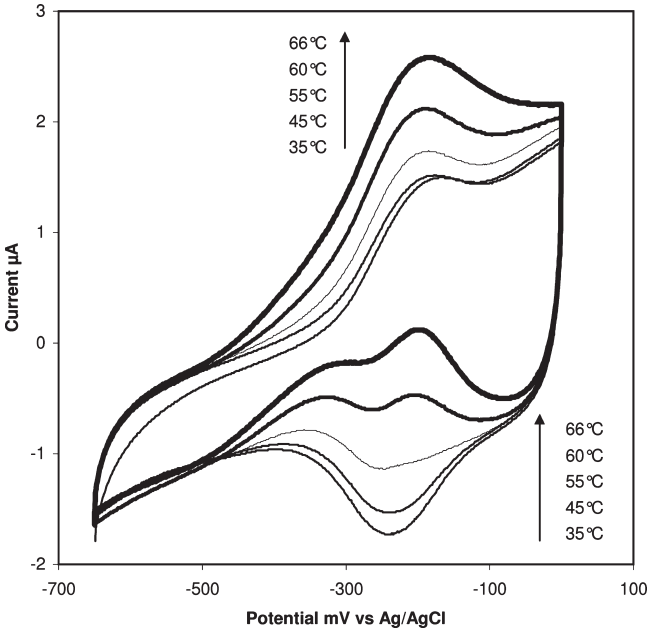


**Figure 3.** Temperature dependence of the CV catalytic $H_2$ oxidation at the MHpTBO electrode; 50 mM HEPES buffer pH 7.2, $H_2$ atm, 5 mV $s^{-1}$; the polymerization process was performed in 160 $\mu$M TBO buffer solution at pH 9.5 for 10 cycles.

protein, with dimensions in the range of a few nanometers. It is hardly possible for it to diffuse through the polymer film. Thus, given that the film thickness is less than 20 nm, the experimental data suggest that electron transfer can occur through the conducting pTBO film.

*$H_2$ Oxidation via Aa Hase Immobilized onto the pTBO Film.* Oxidation of hydrogen by *Aa* Hase entrapped within the membrane at the pTBO-modified PG electrode was followed as a function of temperature. In a first experiment, ten cycles were performed in a 160 $\mu$M TBO Tris-HCl solution pH 9.5 in the −0.65 to +0.9 V range at 50 mV/s. The electropolymerized electrode was then carefully rinsed with water. A total of 1 $\mu$L of 1 $\mu$M *Aa* Hase was then deposited on the surface of the pTBO film. The dialysis membrane was finally deposited onto the biofilm. The so-modified PG electrode, called in the following MHpTBO, was dipped in 50 mM HEPES buffer, pH 7.2 under $H_2$ atmosphere at temperatures from 35 to 66 °C. Figure 3 gathers the characteristic CV curves as the temperature increases. At 35 °C only the redox process for the monomer-like redox couple can be observed at $E_m$ = −220 mV. When the temperature is progressively increased to 66 °C, the anodic current progressively increases, while the cathodic current decreases, describing the characteristic shape for a catalytic oxidation process. A high stability of the electrochemical response is observed. When turning to $N_2$ atmosphere, this catalytic process disappears, and only the redox process for the monomer-like form in the pTBO film is observed. These phenomena reflect $H_2$ oxidation via *Aa* Hase immobilized onto the MHpTBO film.

The thickness of the pTBO film is supposed to greatly affect the catalysis process, essentially by modifying the electron transfer through the redox film. This thickness was thus controlled by varying the number of cyclic scans employed for electropolymerization. The MHpTBO was dipped in 50 mM HEPES pH 7.2 under $N_2$ at 65 °C in order to measure the amount of charge under the pTBO redox peak, thus the film thickness, in the absence of any catalytic process. $N_2$ was then replaced by $H_2$ by flushing $H_2$ into buffer solution for 15 min. CV curves as shown in Figure 4A are consequently obtained.

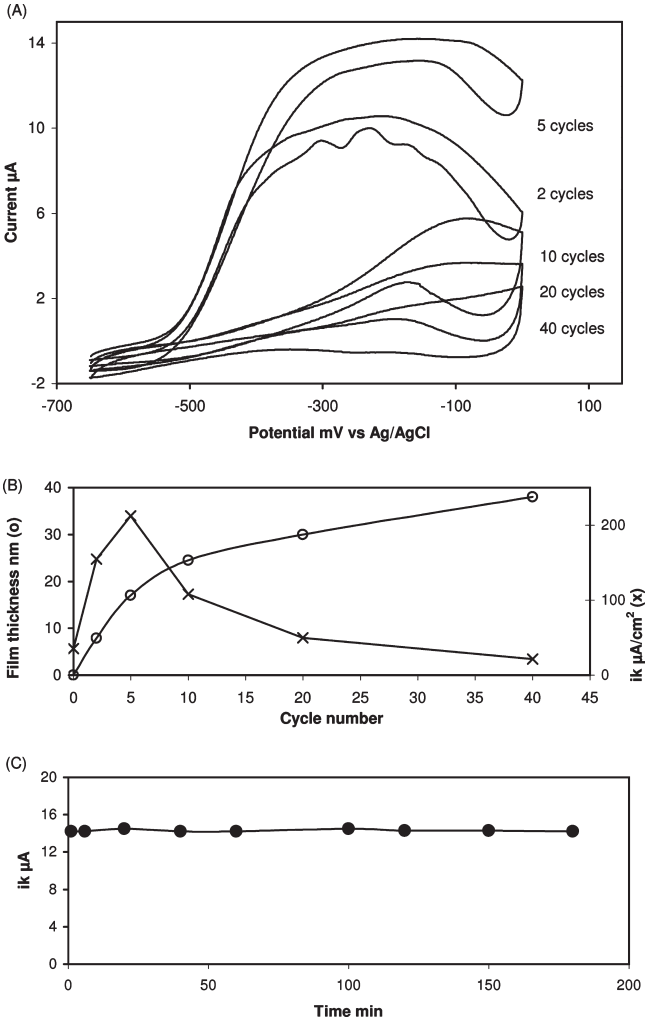


**Figure 4.** (A) CV catalytic $H_2$ oxidation at the MHpTBO electrode dependence on the number of cycles used for electropolymerization; 50 mM HEPES buffer pH 7.2, $H_2$ atm, 65 °C, 5 mV $s^{-1}$; (B) pTBO film thickness and catalytic current (ik) dependence on the cycle number; (C) Evolution of ik with time for a pTBO film realized from 5 electropolymerization cycles; the polymerization process was performed in 160 $\mu$M TBO buffer solution at pH 9.5.

Figure 4B reports the relationship between the catalytic current and the film thickness. The strong dependence of the catalytic response for $H_2$ oxidation by *Aa* Hase on the number of the cyclic scans in the polymerization process is pointed out. Two domains can be defined, with a transition near 5 polymerization cycles.

For the MHpTBO prepared with the lowest numbers of polymerization cycles, the anodic current progressively increases with increasing cycle number, thus film thickness, up to 5 cycles. The presence of the MHpTBO allows a catalytic current 6 times higher than recorded at the bare PG electrode after the same cycling procedure at 60 °C. Other important features can be extracted from the CV signatures: (i) compared to the bare PG electrode, the potential at which $H_2$ oxidation takes place is negatively shifted by 50 mV, as already observed with the addition of TBO in solution, (ii) the catalytic process recorded at the MHpTBO electrode occurs in a wide potential window describing a current plateau from −350 to −100 mV, before *Aa* Hase inactivation, and (iii) a high stability of the electrochemical signal is recorded, with no current loss or potential shift at least for 3 h (Figure 4C). These experimental results demonstrate that the pTBO film provides a very

efficient matrix for *Aa* Hase immobilization. The presence of both the pTBO and the membrane is a prerequisite to provide current stability. The current plateau over a large potential window is attributed to a mixed DET/MET process for $H_2$ oxidation. Indeed, due to the conductivity of the polymer film, the DET process can proceed through the pTBO film involving the anchored *Aa* Hase molecules, but at the same time, the presence of many available redox mediators at the polymer/enzyme interface allows $H_2$ oxidation via a MET process.

When the polymerization scans are increased from 10 to 40 cycles, the catalytic current sharply decreases. The oxidation process potential is now very close to the electrochemical signal for the monomer-like redox couple. It can thus be concluded that for pTBO films thicker than around 20 nm, $H_2$ oxidation via *Aa* Hase mainly proceeds according to a MET process. This is not a very efficient process, however. The lowest conductivity of thick pTBO films, as revealed in this work with the cytochrome $c_3$ study, can be responsible for such a result.

In order to enhance the MET current, an experiment was conducted by electropolymerizing TBO onto a PG electrode coated with SWCN-$CO_2$H. In previous works,[30,31] synergistic effects in the enhancement of NADH oxidation were observed when both carbon nanotubes and pTBO were employed. A bienzyme glucose biosensor was recently described using this architecture.[36] Otherwise, SWCN-$CO_2$H was demonstrated to be a convenient matrix for efficient *Aa* Hase immobilization.[9] In the present work, it was expected that a less compact polymer film, in addition to a higher conductivity brought by the carbon nanotubes, could favor *Aa* Hase interaction with the redox mediator inside the film. The experimental results effectively show an enhanced catalytic current for $H_2$ oxidation, which reaches 425 $\mu$A/cm$^2$ instead of 200 $\mu$A/cm$^2$ in the absence of SWCN-$CO_2$H (Supporting Information, Figure S2). However, loss of electrochemical signal with time is observed. The system thus behaves very much like the one previously described in the absence of pTBO with *Aa* Hase simply adsorbed onto SWCN-$CO_2$H film.[9] The increase in the catalytic signal must be mainly assigned to an enhancement of the surface area due to the carbon nanotube coating.

*Effect of pH Solution for Electropolymerization on the $H_2$ Oxidation Process*. In the preceding experiments, it was noticed that a shift of at least 50 mV toward the negative potentials was recorded for $H_2$ oxidation via *Aa* Hase in the presence of pTBO or TBO in solution. pTBO redox behavior is known to be pH dependent.[37] As a consequence, the catalytic properties of the pTBO film, especially toward NADH oxidation was demonstrated to be also dependent on the pH used for electropolymerization. The pH factor is of course highly crucial when working on hydrogenase systems, as oxidation of $H_2$ produces protons which can modify the local pH, thus affecting the overall catalytic reaction. The effect of the pH of the TBO solution used for pTBO formation on the $H_2$ catalytic oxidation was thus explored. The main result was that no negative shift of the catalytic process was observed when TBO polymerization was performed at a pH value of 4.7 instead of 9.5 (Supporting Information, Figure S3). The p$K_a$ of the amino groups on TBO were evaluated to be around 5.[38] We thus propose that the negative shift observed in the oxidation potential of $H_2$ in the presence of pTBO, could be ascribed to the protonation of the amino groups during $H_2$ oxidation. One key role of the pTBO matrix would be then to maintain a local pH level.

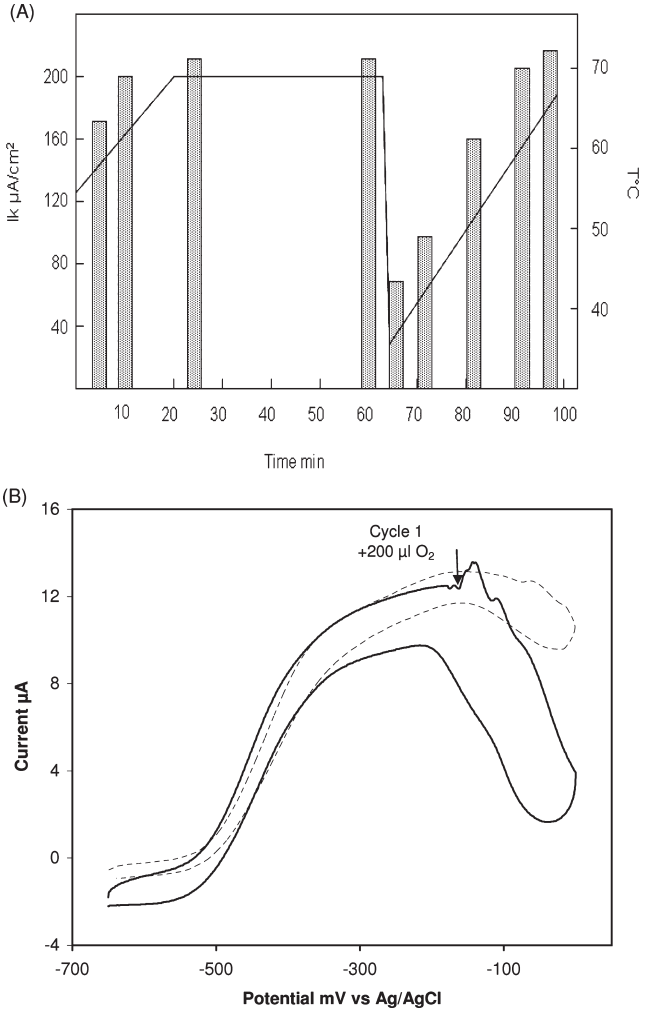


**Figure 5.** Responses to stress of *Aa* Hase entrapped at the membrane pTBO-modified electrode: (A) Temperature dependence of the electrocatalytic current density for $H_2$ oxidation, the continuous line describes the temperature profile and the columns show the values of the catalytic current along with the temperature profile. (B) Influence of $O_2$ addition (indicated by the arrow) on the CV catalytic oxidation of $H_2$ (bold line) at a sweep rate of 5 mV s$^{-1}$, (dotted line) catalytic current after 5 min $H_2$ flushing after cycle 1; 50 mM HEPES buffer pH 7.2, $H_2$ atm., 65 °C; the polymerization process was performed in 160 $\mu$M TBO buffer solution at pH 9.5 for 5 cycles.

*Responses to Stress of Aa Hase Entrapped at the Membrane pTBO-Modified Electrode*. Previous studies emphasized the remarkable tolerance of *Aa* Hase against temperature and inhibitors such as $O_2$ and CO. These characteristics are highly desirable in view of the use of the enzyme in biotechnological devices. *Aa* Hase thus appears as one of the best potential biocatalyst for $H_2$ oxidation. The question arises if such tolerances can be maintained at the pTBO film. This question has been addressed using a pTBO film obtained with the optimal 5 cyclic scans.

The evolution of the catalytic current for $H_2$ oxidation as a function of the temperature at the MHpTBO is shown in Figure 5A.

The catalytic current increases with increased temperatures from 27 to 70 °C. Moreover, when the temperature is decreased down to 20 °C after 1 h of storage at 70 °C, and increased again at 60 °C, the value of the catalytic current is restored. This remarkable evolution suggests a quite stable protein conformation in a wide range of temperature.

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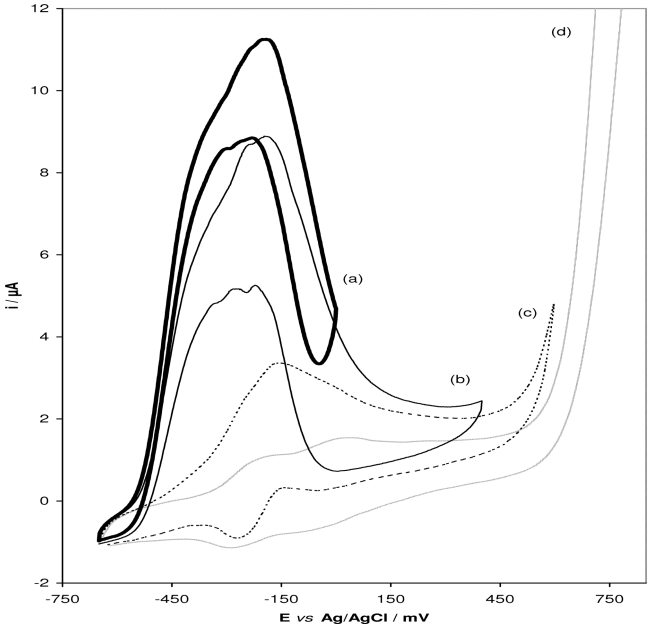


**Figure 6.** Anodic potential dependence of the CV catalytic $H_2$ oxidation via *Aa* Hase in a thin layer at the membrane PG electrode; (a) −0.65 to 0 V, (b) −0.65 to +0.4 V, (c) −0.65 to +0.6 V, (d) −0.65 to +0.9 V; 16 $\mu$M TBO in 50 mM HEPES buffer pH 7.2, 65 °C, $H_2$ atm, 5 mV $s^{-1}$.

To test $O_2$ resistance we used the method previously developed by Vincent et al. to investigate $O_2$ inhibitory effect onto [NiFe]-Hases.[39] During CV recordings at the MHpTBO electrodes, 200 $\mu$L of $O_2$-saturated buffer solutions were injected into the 3 mL cell solution (around 105 $\mu$M final $O_2$ concentration). $O_2$ was injected at approximately −200 mV. The CV curve given in Figure 5B (bold line) indicates that the CV current drops after $O_2$ exposure, but is almost recovered on the reverse scan. Furthermore, 5 min of hydrogen flushing allows a total recovering of the catalytic current for $H_2$ oxidation (dotted line). It can be concluded that the intrinsic tolerance against $O_2$ of *Aa* Hase is preserved at the MHpTBO electrode.

*Essays for Entrapment of Aa Hase into the pTBO Film.* Enzyme/polymer composite electrodes were classically prepared by cyclic potential sweep in the polymerization potential range in buffer containing both the dye and the enzyme. This procedure succeeded especially in the case of glucose oxidase[32] or horseradish peroxidase.[40] In this work immobilization of *Aa* Hase into the pTBO film by entrapment of the enzyme during the electropolymerization procedure failed. This is clearly demonstrated in figure 6.

*Aa* Hase is immobilized at the PG membrane electrode and dipped in a 16 $\mu$M TBO HEPES solution, at pH 7.2. This pH is chosen to conduct electropolymerization under soft conditions for enzyme stability. Under $H_2$ atmosphere at 65 °C, the typical catalytic signal for hydrogen oxidation via *Aa* Hase is obtained. A mixed DET/MET process is observed as detailed in the first part of this work. The limiting anodic potential is then progressively shifted from 0 to 0.9 V. As a consequence the catalytic oxidation signal dramatically decreases. When the limiting upper potential is above 0.5 V, only the signal of pTBO is revealed, thus indicating that *Aa* Hase is no longer efficient.

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## 4. Conclusion

In this work we have shown that $H_2$ oxidation process by the hydrogenase from the extremophilic bacterium *Aquifex aeolicus* is similar at a membrane electrode configuration than at a bare PG electrode. This suggests the involvement of adsorbed *Aa* Hases in the DET process at the membrane electrode. From cyclic experiments, it appears that the decrease in enzymatic activity observed with time is not simply related to enzyme leaching. Other processes might be considered, including protein denaturation or reorientation. This point is still under investigation in our laboratory, especially with the aids of surface spectroscopies. Nevertheless, we have demonstrated that the presence of a phenothiazine dye in the membrane configuration offers one solution for solving this problem. Actually, *Aa* Hase activity at the membrane PG electrode with TBO in solution shows two processes highly stable in time, i.e., direct and mediated electron transfer processes for $H_2$ oxidation. The MET process is not unexpected since TBO is available in excess to connect the *Aa* Hase molecules possibly released from the electrode but entrapped in the thin layer. The concomitant increase in the DET process implies that TBO acts not only as a redox mediator. On adsorbed TBO the two DET and MET processes occur, showing that the TBO film plays the role of an efficient anchor for hydrogenase immobilization. *Aa* Hase immobilization on electropolymerized pTBO film yields a high and stable $H_2$ oxidation activity at the membrane electrode, as long as film thickness stays below 20 nm. TBO either in solution or electropolymerized at the PG electrode induces an increase in current and a negative potential shift of about 50 mV of the DET process. Favorable contacts between amino acid residues that are exposed to solvent and TBO molecules through hydrophobic and/or electrostatic interactions might be involved in these improvements of the catalytic signal, as a consequence of *Aa* Hase anchorage. But it must be reminded that high stability of the electrochemical signal for $H_2$ oxidation was also recently demonstrated using *Aa* Hase encapsulation into carbon nanotube networks.[9] pTBO and carbon nanotube networks share an electron-rich surface, and this could also be a key point in the catalytic signal stability. In this latter case, the matrix would play the role of ROS scavenger, by driving oxygen trace reduction to water. Buffering capability of the polymer matrix to drive pH local changes is finally underlined. This is an important issue in view of the development of biotechnological devices, where the design of the fuel cell imposes to evacuate the $H_2$ oxidation products.

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**Supporting Information Available:** Stability and conductivity of the pTBO film (Figure S1), comparative CV curves for $H_2$ oxidation by *Aa* Hase at pTBO- and (pTBO+SWCN)-modified PG electrode (Figure S2), and effect of the pH used for electrolymerization on the catalytic process (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.