NEW HYPOTHESES FOR HYDROGENASE IMPLICATION IN THE CORROSION OF MILD STEEL

Maha MEHANNA[a], Régine BASSEGUY[a], Marie-Line DELIA[a], Laurence GIRBAL[b], Marie DEMUEZ[b], Alain BERGEL[a, *]

[a] *Laboratoire de Génie Chimique (LGC) CNRS-INPT, 5 rue Paulin Talabot BP 1301, 31106 Toulouse, France*

[b] *Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés (LISBP) CNRS-INSA, 135 Avenue de Rangueil, 31077 Toulouse, France*

ABSTRACT

The influence of [Fe]-hydrogenase from *Clostridium acetobutylicum* was studied on the anaerobic corrosion of mild steel. Two short-circuited mild steel electrodes were exposed to the same solution and hydrogenase was retained on the surface of only one electrode thanks to a dialysis membrane. The galvanic current and the electrode potential were measured as a function of time in order to monitor the difference in electrochemical behaviour induced by the presence of hydrogenase. A sharp potential decrease of around 500 mV was controlled by the deoxygenating phase. When hydrogenase was introduced after complete deoxygenation, significant heterogeneous corrosion was observed under the vivianite deposit on the electrode in contact with hydrogenase, while the other electrode only showed the vivianite deposit,

[*] Alain.Bergel@ensiacet.fr, Phone : +33534615248, Fax : +33534615253

which was analysed by MEB and EDX. The effect of hydrogenase was then confirmed by monitoring the free potential of single coupons exposed or not to the enzyme in a classical cell after complete deoxygenating. In both phosphate and Tris-HCl buffers, the presence of hydrogenase increased the free potential around 60 mV and induced marked general corrosion. It was concluded that [Fe]-hydrogenase acts in the absence of any final electron acceptor by catalysing direct proton reduction on the mild steel surface.

Keywords : Hydrogenase; Anaerobic; Biocorrosion; Mild steel; Phosphate.

1. Introduction

Corrosion costs 4% of the GNP of industrialized countries out of which 10% are due to biocorrosion [1]. It is now commonly agreed that sulfate-reducing bacteria and thiosulfate reducing bacteria (SRB/TRB) are the main causes of anaerobic Microbially Influenced Corrosion (MIC) [2-6]. Many mechanisms have been proposed to explain anaerobic MIC by SRB and TRB: precipitation of iron sulphide, which next catalyses proton reduction into molecular hydrogen and acts as a cathode in a galvanic couple with metallic iron; anodic depolarization resulting from the local acidification at the anode [7]; possible production of corrosive phosphides containing metabolite PH_3 that enhances the dissolution of metal under anaerobic conditions [8]; metal ion complexation by extra cellular polymer substances [9]; diminution of the pH and metabolism of reducing thiosulfate to sulphide [10].

The involvement of hydrogenases which are either present in bacteria or free in solution remains subject to many debates. While it has been claimed that a hydrogenase negative strain of SRB was more corrosive than hydrogenase positive strains [11], other studies have demonstrated that there is a direct correlation between the presence of hydrogenase in SRB and corrosion [12]. The same authors found that hydrogenase increased the corrosion rate when used in a phosphate solution and proposed the following chemical reaction between steel and phosphate ions [13]:

$$3Fe° + 4H_2PO_4^- \longrightarrow Fe_3(PO_4)_2 + 3H_2 + 2HPO_4^{2-}$$

Also, the ability of hydrogenases from *T. roseopersicina* and *L. modestohalophilus* to oxidize metals even without the need for a mediator has been confirmed [14]:

$$M \xrightarrow{HASE} M^{n+} + ne\text{-}.$$

Moreover, it has been claimed that [Ni-Fe]-hydrogenase from *Ralstonia eutropha* is an effective trigger of mild steel corrosion when used in phosphate solution with no need for a mediator [15-16].

Hydrogenases are enzymes that catalyse the reversible oxidation of molecular hydrogen:

$$H_2 \xleftrightarrow{HASE} 2H^+ + 2e^-$$

They are divided into 3 groups according to the composition of their active site: [NiFe], [Fe] and transition metal free hydrogenases [17-18]. The [NiFe] and [Fe] hydrogenases constitute the vast majority and both contain a binuclear metal active site. The [NiFe] hydrogenases have a minimum of two subunits: the catalytic site that contains the active site, and the electron transferring subunit that contains one or more iron-sulfur centres. The [Fe]-hydrogenases may be constituted by only one subunit, which may include the catalytic and electron transferring domains [19]. [Fe]-hydrogenases are known to have 100 times more H_2 production specific activity than [NiFe]-hydrogenases [20], therefore a [Fe]-hydrogenase

from *Clostridium* was chosen here, suspecting that it might be more effective in MIC than the [NiFe]-hydrogenase studied previously. The aim of this work was to determine the possible influence of this enzyme on anaerobic MIC of mild steel. In the first phase of the study, two short-circuited mild steel electrodes were exposed to the same phosphate solution and hydrogenase was retained on the surface of only one electrode thanks to a dialysis membrane. It was expected that the galvanic current between both electrodes gives a measure of the electrochemical disturbance induced by the presence of hydrogenase on the surface of one electrode only. In the second phase, experiments were conducted with a single electrode in order to avoid any disturbance due to the deoxygenating phase. The influence of the buffer solutions phosphate and Tris-HCl and possible mechanisms were discussed. The surface deposits on the electrodes were examined and the influence of the buffer was also investigated.

2. Experimental

2.1. Chemicals and biochemicals

Sodium dihydrogenophosphate was purchased from Prolabo, tris(hydroxymethyl) aminomethane from Acros Organic, sodium dithionite and desthiobiotine from Sigma. *Clostridium acetobutylicum* cultures and hydrogenase preparation were carried out at the LISBP as reported elsewhere [21].

2.2. Electrochemical measurements

Working electrodes were 2 cm diameter cylinders of XC45 mild steel purchased from Thyssen (elemental composition by weight percentage: 0.46 C, 0.31 Si, 0.65 Mn, 0.01 P, 0.032 S, 0.1 Cr, 0.1 Ni, 0.02 Mo, 0.05 Al, 0.11 Cu) embedded in resin (Resipoly Chrysor). Electrical connection was made through titanium wire protected with resin. Coupons were polished successively with SiC papers of P120, P180, P400, P800, P1200, P800/2400, P1200/4000 grit (Lam Plan) and rinsed thoroughly with distilled water.

The galvanic cell was composed of two compartments separated by a dialysis membrane (Cellu Sep T4 12-14kDa) as shown in Fig. 1. Compartment A, where the enzyme was injected, and B had volumes of 10 and 60 mL respectively. Electrodes were put face to face in the galvanic cell and coupled through a Keithley 2000 picoamperemeter. The dialysis membrane confined the enzyme near the surface of only one electrode (compartment A) while both electrodes were exposed to the same solution. Oxygen was removed by continuous nitrogen flow in compartment B. The potential of the electrode of the enzyme compartment was measured versus a saturated calomel electrode (SCE). Both electrodes had the same potential because they were connected in short-circuit mode through the picoampermeter which had a null resistance.

Experiments without coupling were performed in a classic Metrohm cell. The open circuit potentials E_{corr} were measured with a multipotentiostat Ec-Lab with respect to a SCE reference.

3. Results and discussion

Two identical XC45 mild steel electrodes were set up in the galvanic cell and coupled through a picoamperemeter. Both compartments A and B were filled with 100 mM phosphate

solution pH 8.0, and oxygen was removed by a continuous nitrogen flow in compartment B. The potential recorded as a function of time (Figure 2.A) always showed an abrupt decrease by 500 mV that occurred at different times. The galvanic current (Figure 2.B) firstly fluctuated between positive values (maximum +2.2 µA cm^{-2}) and decreased in most of the time to negative values after 15 hours (minimum value -1.36 µA cm^{-2} after 24 hours). The positive values indicated that electrons flowed in the external electrical circuit from electrode B to A, which means that electrode A acted as a cathode. At the end of the experiences, both electrodes appeared visually similar: they were covered by a light gray non conductive film that turned into bluish-green with exposure to air. SEM pictures indicated that this deposit was uniform on the whole surface (Figure 3), and by EDX analysis, we assumed that it was vivianite ($Fe_3(PO4)_2$, $8H_2O$). Previous studies carried out in phosphate buffer solutions have also observed such a deposit, which is known to protect against corrosion [22].

A series of five experiments was conducted by injecting 23 U mL^{-1} hydrogenase from *Clostridium acetobutylicum* in the compartment A. Hydrogenase was introduced at 1 hour, before the potential decreased (Figure 4). In two cases, the galvanic current got positive values in the same range as in the absence of hydrogenase. This behaviour was similar to that observed in the absence of the enzyme. On the contrary, in three other cases the current decreased toward more negative value than in the absence of hydrogenase: a minimum value around -5.7 µA cm^{-2} was obtained. After 24 hours, both electrodes were covered by a vivianite deposit, which was thicker on the coupon from compartment A. Actually, the thickness of the vivianite deposit was roughly evaluated by polishing the coupons, and it was significantly harder and longer to polish the electrodes from compartment A than the electrodes from compartment B.

Finally, when hydrogenase was injected at time 15 minutes, but after the potential decreased (Figure 5), the current was in majority positive, up to + 2.6 µA cm^{-2} and then decreased to

zero or slightly negative values. After removing the electrodes from the galvanic cell, in addition to the bluish-green vivianite deposit, deep pits were observed on the electrodes from compartment A. After polishing these electrodes with the SiC papers to remove the vivianite deposit, the pits remained visible as shown in figure 6.A'. No pits were detected on the electrodes from the compartment B that did not contain the hydrogenase, neither before nor after polishing. Only a uniform vivianite deposit was observed.

In order to avoid the bad controlled potential drop, which was certainly due to the deoxygenating phase, experiments were performed with only one coupon in a classic electrochemical cell in phosphate buffer 10 mM pH 8.0 instead of 100 mM in order to slow down the deposition of vivianite which protects from corrosion. Evolution of the free potential with respect to a SCE reference was recorded as a function of time (Figure 7). When the nitrogen flow was put on only after 20 hours (experiment 1), the free potential fluctuated in a large range and started to decrease definitively after 21 hours until it reached -0.76 V versus SCE at 24 hours. When a strong nitrogen flow was put on just at the beginning of the experiment (experiments 2 and 3), the potential dropped down in a few minutes to the lowest value. It remained stable around -0.77V versus SCE in experiment 3. In experiment 2 the nitrogen flow was stopped after 2 hours, and the free potential increased continuously back to the initial value around -0.27 V versus SCE. When the nitrogen flow was put on, 30 minutes before submerging the working electrode (experiments 4 and 5), the potential got to its lowest value since the beginning and remained stable nearby -0.76 V versus ECS. It fluctuated a little whenever any change in the bubbling occurred.

These experiments confirmed that the potential was directly controlled by the deoxygenating process. It can be suspected that in the previous experiments traces of oxygen remained in compartment A, and the differences observed in the times of the potential decrease corresponded to the time that was required to deoxygenate completely this compartment

through the dialysis membrane. The hydrogenase from *C. acetobutylicum* is highly sensitive to any trace of oxygen [23], consequently when it was injected before the potential decrease, it was certainly inactivated and logically no significant effect should be observed on the galvanic current as was the case in two experiments in Figure 4. Nevertheless, the presence of hydrogenase had an effect on the current in three cases and it favoured thicker vivianite deposit. This may be due to a possible residual activity of the protein. A possible involvement of the iron-sulfur clusters contained in the hydrogenase (five [Fe-S] per enzyme molecule), or released from it, may also be suspected.

Introducing the hydrogenase in compartment A after complete deoxygenating protected its activity, and clear effects were so observed in Figure 5. The negative values of the current mean that the electrode in compartment A globally behaved like an anode, positive values signify that it was globally a cathode. The fluctuation between positive and negative values suggests that hydrogenase favoured both anodic and cathodic sites on the same electrode, its global behaviour being controlled by the balanced between the anode and cathode local sites. Such local anode/cathode sites on steel surfaces have already been evoked in the presence of hydrogenase from *Ralstonia eutropha* [22]. The most obvious corrosion features (Figure 6) were obtained when the potential dropped very fast at the beginning of the experiment, *i.e.* when oxygen was removed very fast from the beginning and hydrogenase was introduced without delay. In this case, hydrogenase provoked obvious local corrosion pits under the vivianite deposit. Such a strong sign of corrosion has not been observed previously with the hydrogenase from *R. eutropha*. From this view, it should be concluded that the [Fe]-hydrogenase from *C. acetobutylicum* seems more dangerous in triggering corrosion than the NAD-dependent [NiFe]-hydrogenase from *R. eutropha*.

The differences observed according to the injection time are still difficult to explain. Actually, several antagonist phenomena were combined in these experiments, the initial differential oxygenation between compartments A and B, the different stages in the vivianite deposit formation at the time when hydrogenase was introduced, completed or uncompleted enzyme denaturing, iron-sulfur cluster release, etc. From now on, the experiments were conducted in a simpler way, with only a classic electrochemical cell to avoid irreproducibility due to differences in the time of oxygen removal. The electrode was first maintained above the solution, and the solution was deoxygenated by strong nitrogen bubbling during 30 minutes. The electrode was then plunged into solution and continuous nitrogen flow was kept on during all the experiment in order to avoid any oxygen contamination. The potential was allowed to stabilize for 15 minutes and hydrogenase 2.5 U mL^{-1} was then injected. The evolution of the free potential as a function of time showed a continuous increase of the potential, which could reach 50 mV after 24 hours, due to the addition of the hydrogenase (Figure 8).

After 24 hours, the electrodes in the presence of hydrogenase showed significant general corrosion in addition to a thick vivianite deposit. After removing the vivianite deposit by polishing, pits of heterogeneous corrosion were also observed. Electrodes in the absence of hydrogenase were only covered by a vivianite layer (Figure 9).

For enzymatic H_2 production, the *C. acetobutylicum* hydrogenase is generally implemented in 100 mM Tris HCl buffer pH 8.0, with 150 mM NaCl, 2 mM sodium dithionite and 2,5 mM desthiobiotine. Preliminary experiments showed that this medium was highly corrosive because of the presence of NaCl and the compounds added to stabilize the enzyme (destiobiotine) and to protect it from oxygen (dithionite). These compounds were consequently removed, taking great care to avoid any contact of the enzyme with oxygen, and using only enzyme samples freshly prepared. Experiments were done in the classical

electrochemical cell using 50 mM of Tris HCl pH 6.3. This pH value is generally used to favour H_2 production. When 4.2 U mL^{-1} hydrogenase was added, the potential increased fast following the injection, up to 70 mV (Figure 10). After 24 hours, general corrosion showing a reddish deposit was clearly present on the coupons in contact with hydrogenase (Figure 11).

Corrosion of carbon steels induced by the presence of hydrogenase has already been reported by different authors. Most previous experiments have been implemented in phosphate solutions, and phosphate species have been demonstrated to be directly involved in corrosion enhancement through direct deprotonation on the steels surfaces as in scheme A of Figure 12 [24]. The cathodic deprotonation reaction produces dihydrogen in a reversible way [7] and the hydrogenase enhances corrosion by consuming the dihydrogen produced. For this scheme be effective, the presence of a final electron acceptor (e.g. an organic dye [13] or NAD^+ [24]) is required. On the contrary, there was no such final electron acceptor in the experiments reported here, and this scheme cannot be evoked. Moreover, the last series showed strong corrosion in the absence of phosphate species. As a conclusion, the presence of phosphate species was not required here for corrosion to occur, on the contrary its presence made the process more complex because of the deposit of protective compounds like vivianite. The sole mechanism that can explain the corrosion observed here is the catalysis by adsorbed hydrogenase of the direct reduction of proton or solvent, as schematized in Figure 12.B. This mechanism has already been demonstrated by electrochemical measures and it has been suspected to be able to enhance corrosion of steels but, to our knowledge, this was here the first time that it was proved to be able to provoke actual corrosion.

No such clear demonstration has been obtained yet with the NAD-dependent [NiFe]-hydrogenase from *R. eutropha*. *C. acetobutylicum* [Fe]-hydrogenase and *R. eutropha* [NiFe]-hydrogenase share some resemblances in the H_2-activating bi-nucleic centers as shown by infrared spectroscopy [25], and the amino acid sequences encoding the binding sites of at

least two [4Fe-4S] clusters are alike. Nevertheless, *C. acetobutylicum* [Fe]-hydrogenase has less than 23% homology in amino acid sequences with each of the four subunits of *R. eutropha* NAD-dependent [NiFe]-hydrogenase. In addition, the molecular organization and the nature of the clusters are different in each enzyme [26], and [Fe]-hydrogenases are known to be around 100 times more effective for H_2 production than [NiFe]-hydrogenases [20]. It may consequently be suggested that [Fe]-hydrogenases may be more active in corrosion than other types of hydrogenase and/or that a correlation exists between corrosion risk and hydrogenase activity for hydrogen production.

4. Conclusion

Two mechanisms have been suggested in the literature to explain the involvement of hydrogenase in corrosion of steels. One is based on the cathodic deprotonation of phosphate species, which is reversible and can so be accelerated by the hydrogenase-catalysed consumption of the dihydrogen produced. The other assumed the direct catalysis of proton/solvent reduction by adsorbed hydrogenase. Here the [Fe]-hydrogenase from *Clostridium* was revealed highly efficient according to the second mechanism. To our knowledge, it was the first clear demonstration that a free hydrogenase can enhance corrosion in the absence of phosphate species and in the absence of any final electron acceptor, it means in conditions similar to the real conditions that can be encountered inside natural biofilms.

As a conclusion, this study established the efficiency of hydrogenase in enhancing corrosion through the direct catalysis of proton/water reduction, and it suggests that the intrinsic activity of hydrogenases may be a key parameter with the view to assess biocorrosion risks.

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Fig. 1. Scheme of the galvanic cell.

Fig. 2. Evolutions of the potential (A) and the galvanic current (B) as a function of time obtained with two XC45 coupled electrodes in a 100 mM phosphate solution pH 8.0, under deoxygenation but without hydrogenase addition.

Fig. 3. SEM analysis and EDX results for (A, A') XC45 electrode after polishing and (B, B') XC45 electrode after 24 hours immersion in 100 mM phosphate solution pH 8.0, under deoxygenation but without hydrogenase addition.

Fig. 4. Evolutions of the potential (A) and the galavanic current (B) as a function of time obtained with two XC45 electrodes in a 100 mM phosphate solution pH 8.0 in the presence of 23 U mL^{-1} hydrogenase in compartment A. Hydrogenase was injected at 1 hour, before the potential decreased.

Fig. 5. Evolutions of the potential (A) and the galvanic current (B) as a function of time obtained with two XC45 electrodes in a 100 mM phosphate solution pH 8.0 in the presence of 23 $U.mL^{-1}$. Hydrogenase was injected at 15 minute, after the potential decreased.

Fig. 6. Pictures of the XC45 carbon steel electrodes from the experiment reported in Figure 5: 100 mM phosphate solution pH 8.0, hydrogenase 23 U mL^{-1} in compartment A, injected at 15 minute, after the potential decreased. (A) Electrode from compartment A. (A') Same electrode after polishing with SiC papers. (B) Electrode from compartment B, without enzyme.

Fig. 7. Free potential as a function of time for XC45 carbon steel electrodes in 10 mM phosphate solution pH 8.0, with deoxygenating at different times.

Fig. 8. Free potential as a function of time for XC45 carbon steel electrode in 100 mM phosphate solution pH 7.2, with or without addition of hydrogenase. Fluctuations of +/-10 mV that appear on the graph each four hours are due to polarization resistance measurements.

Fig.9. SEM pictures of XC45 carbon steel electrodes after 24 hours in 100 mM phosphate solution pH 7.2. (A) with 2.5 U mL^{-1} hydrogenase. (B) without hydrogenase.

Fig.10. Free potential as a function of time for XC45 carbon steel electrode in Tris HCl 50 mM, pH 6.3, with or without hydrogenase added.

Fig. 11. Pictures of XC45 carbon steel electrodes after 24 hours in Tris HCl 50 mM, pH 6.3. (A) with 4.2 U mL^{-1} hydrogenase. (B) without hydrogenase.

Fig. 12. Possible cathode reactions catalysed by hydrogenase that may enhance corrosion.