

ACTIVATION, REDUCTION AND PROTON-DEUTERIUM EXCHANGE REACTION OF THE PERIPLASMIC HYDROGENASE FROM *DESULFOVIBRIO GIGAS* IN RELATION WITH THE ROLE OF CYTOCHROME c_3

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1. Introduction

The enzyme hydrogenase catalyzes the reversible oxidoreduction of the dihydrogen molecule, according to the reaction: $H_2 \rightleftharpoons 2H^+ + 2e^-$ [1]. Depending on the microorganisms and on the environmental conditions [2] the overall balance of the reaction can be directed towards either the production or the consumption of hydrogen gas. With the purified enzyme it is always possible with an adequate electron donor or acceptor to let the reversible reaction go in one or the other direction but the activity exhibited strongly depends upon the nature of the redox agent, especially its potential, and of the medium conditions [3].

The H^+ - D_2 (or D^+ - H_2) exchange reaction is part of the reversible activity of hydrogenase [4] and provides an intrinsic measure of this activity. Since the overall balance is nil the exchange should proceed in the absence of any electron donor or redox substance. Yet, whereas the reaction readily takes place with the living cells [5], crude extracts [6] or even partly purified hydrogenases [7], the isolated enzyme in contrast has to be first activated for the exchange to proceed. This is generally achieved by addition of dithionite [6] or, in the case of *Desulfovibrio*, of the specific cytochrome c_3 [8]; however both agents may interfere in several ways with the exchange reaction. The problem arises from the involvement in the loss and recovery of hydrogenase activity of different processes such as oxidation and reduction, oxygenation and deoxygenation. Dithionite for instance scavenges oxygen from the medium and from the enzyme centers and at the same time reduces hydrogenase [6]. Moreover, its action is altered according to the pH conditions [9].

As for cytochrome c_3 it can act once reduced as a simple oxygen scavenger or, as it had been reported for *Desulfovibrio vulgaris* Miyazaki [8,10], it can accelerate both H_2 evolution and the exchange reaction. On the contrary such a stimulation of H_2 evolution has not been observed with *Desulfovibrio gigas* hydrogenase after addition of cytochrome c_3 from that same organism [11]. It was therefore of interest to check whether in the case of the latter species cytochrome c_3 had an effect upon the exchange reaction.

This work shows that with the purified periplasmic hydrogenase from *D. gigas* the addition of an electron carrier or of a reducing agent was unnecessary either for the exchange reaction or for the activation of the enzyme. In the latter process, two successive steps were observed:

- (1) Requiring that oxygen be excluded from the enzyme centers but also probably implying a modification in these centers; this step could be achieved by physical or chemical means and was only accelerated in the presence of dithionite or cytochrome c_3 .
- (2) Consisting in a spontaneous reduction of the enzyme under molecular hydrogen and following the same kinetics whether cytochrome c_3 was present or not.

2. Materials and methods

The periplasmic hydrogenase of *D. gigas* was isolated and purified to homogeneity as in [11].

The exchange between D_2 and H^+ was followed directly in the liquid phase thanks to a reaction vessel

connected to a mass-spectrometer via a teflon membrane allowing the diffusion of dissolved gases to the ion-source [12]. In short, 10 ml 0.05 M Tris-HCl buffer (pH 7.6) contained in the vessel (thermostatted at 30°C) were sparged till saturation with a gas mixture Ar + D₂. The gas phase was then eliminated and the vessel was closed after which an aliquot of hydrogenase preparation was injected anaerobically.

The ensuing exchange reaction, involving the quantitative replacement of D₂ by HD then H₂, was monitored by scanning mass-peaks 4,3 and 2 over brief intervals. This very sensitive method allows continuous measurements with small enzyme samples over short periods. The level of dissolved oxygen (mass-peak 32) was also checked at the beginning and at the end of each experiment. According to the experimental purpose, dithionite, cytochrome *c*₃ or an auxiliary oxygen scavenging system (glucose + glucose oxidase) could also be introduced into the vessel.

The activation of the hydrogenase was carried out in anaerobic spectrophotometer cuvettes containing 1 μ M hydrogenase in 3 ml of the same Tris-HCl buffer under a hydrogen flow. The redox status of the enzyme was checked periodically by recording the optical spectrum from 350–625 nm (Aminco-Chance DW2A spectrophotometer). At the same time a 50 μ l aliquot was sampled from the cuvette and injected into the reaction vessel for the mass-spectrometric measurement of the exchange rate.

3. Results

The spectra of the oxidized and of the reduced enzyme were recorded to check the absence of cytochrome *c*₃ (fig.1). Although hydrogenase was in both cases initially oxidized, the activation pattern before the exchange reaction was effective differed whether the enzyme was freshly prepared or had been stored for some time.

With the freshly isolated enzyme the activation occurred within a few minutes in the reaction vessel. The exchange activity in that case was monitored as the D₂ disappearance kinetics (fig.2). In the presence of dithionite, the exchange reached its maximal rate immediately upon injecting hydrogenase into the vessel (fig.2e). Without dithionite but with diverse amounts of cytochrome *c*₃ from the same species a lag was observed, the longer the lower the cytochrome concentration, after which the exchange rate gradu-

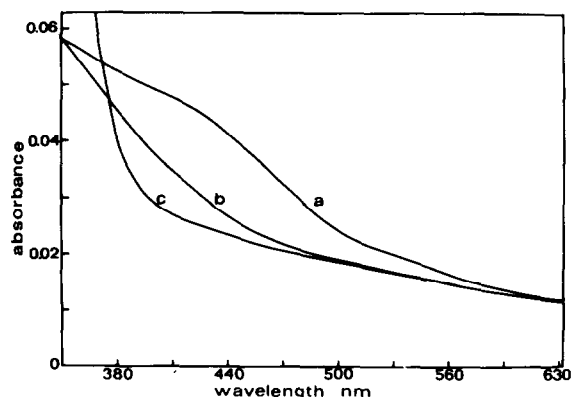


Fig.1. Spectra of oxidized and reduced periplasmic hydrogenase from *Desulfovibrio gigas*: (a) oxidized hydrogenase (1 μ M); (b) hydrogenase reduced by H₂ (flushed for 2 h); (c) hydrogenase reduced by a slight excess of Na₂S₂O₄.

ally increased (fig.2d,c). In the absence of both dithionite and cytochrome *c*₃ a steady exchange rate was reached still more slowly (fig.2b). When glucose plus glucose oxidase were present the kinetics pattern approached that obtained with dithionite (not shown), indicating that the exchange reaction depended on

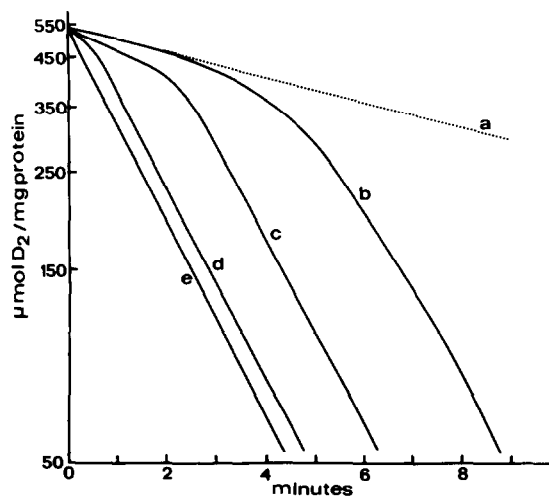


Fig.2. Exchange kinetics of freshly prepared periplasmic hydrogenase from *D. gigas*. A hydrogenase sample was injected at time 0 into the reaction vessel previously sparged with a mixture Ar + D₂ (8:2, v/v) then the deuterium partial pressure was recorded (semilogarithmic plot): (a) no hydrogenase added (mass-spectrometer self-consumption); (b) hydrogenase alone (final conc. 2.5 nM); (c) hydrogenase plus *D. gigas* cytochrome *c*₃ (1 nM); (d) hydrogenase plus cytochrome *c*₃ (10 nM); (e) hydrogenase plus Na₂S₂O₄ (27 μ M).

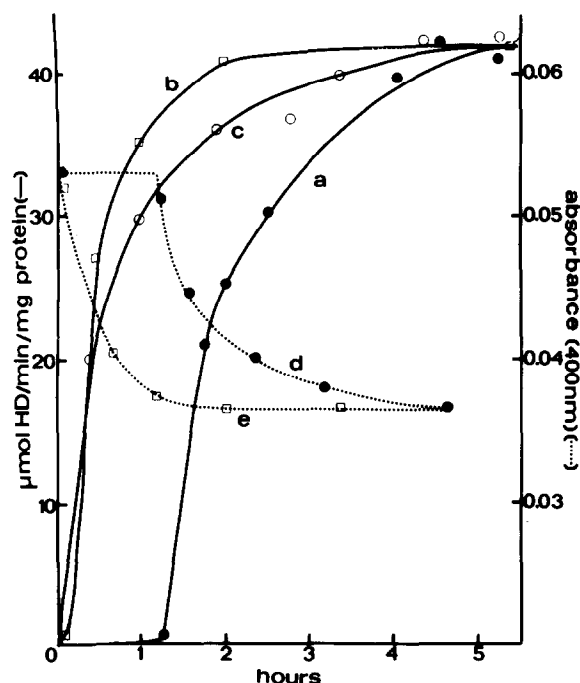


Fig.3. Hydrogenase reduction and reactivation: 3 ml spectrophotometer anaerobic cuvettes containing $1 \mu\text{M}$ periplasmic hydrogenase from *D. gigas* were flushed with pure H_2 from time 0 onwards. At intervals the exchange activity (initial HD production, —) and the absorbance at 400 nm (---) were measured. (a,d) Cuvette directly flushed with H_2 ; (b,e) cuvette flushed for 2 h with Ar then transferred to H_2 at time 0; (c) cuvette flushed with H_2 after addition of cytochrome c_3 from *D. gigas* (final conc. 27 nm).

oxygen removal. In all instances, the exchange rate tended towards the same velocity constant after a few minutes.

In contrast, hydrogenase stored for some months in liquid nitrogen had to be reactivated by flushing hydrogen or deuterium for a fairly long time before the exchange activity was exhibited. After that lag-time the exchange rate (measured here as the initial HD production velocity following each enzyme introduction) increased till a limit value (fig.3a) while the enzyme was concomitantly reduced (fig.3d). Neither activation nor reduction were observed when the enzyme was flushed with pure argon instead of hydrogen. However, when the enzyme pre-flushed with argon was transferred to hydrogen, the lag-time under H_2 was considerably shortened but afterwards, the activation (fig.3b) and reduction (fig.3e) kinetics were not sensibly modified. When cytochrome c_3 from *D. gigas* was added it was almost immediately

reduced to the contact of hydrogen and hydrogenase after which the activation of hydrogenase began without any lag but followed the same kinetics and reached the same level (fig.3c). A similar pattern was observed with dithionite (not shown).

4. Discussion

Activation of the periplasmic hydrogenase of *D. gigas* consists of at least two steps:

- (1) Corresponds to the lag-time elapsed before the appearance of any exchange activity. Here, hydrogen could be replaced by argon;
- (2) In contrast, the presence of hydrogen was required to reduce the enzyme whilst the exchange rate was increasing.

With the freshly prepared enzyme both steps were more or less overlapping and the exchange velocity increased gradually and reached rapidly a limit value. In agreement with [6] such an activation pattern can be explained by a rapid exclusion of oxygen from the active centers of the enzyme. In the absence of an oxygen scavenging system this was achieved by the oxyhydrogen reaction (O_2 traces eventually present actually disappeared during the experiment, the lag-time being then the longer). This reaction was faster in the presence of cytochrome c_3 , dithionite or glucose plus glucose oxidase.

When hydrogenase had been stored for some time the 2 steps were clearly distinct and their duration depended on the inactivation degree of the enzyme. In fig.3 for instance, with an enzyme sample stored 1 year, the first step lasted $>1\text{h}$. Such a long and moreover variable duration cannot be fully explained by a mere oxygen removal under the hydrogen or argon flow but rather by the dissociation of an enzyme-oxygen combination [6] or by a change in the enzyme active centers following oxygen removal.

The exchange activity was not recovered in that first step and hydrogenase had to be further reduced by hydrogen. This result seems to be conflicting with a report according to which the activation of the hydrogenase from *Methanobacterium* strain G2R required the presence of either dithionite or glucose + glucose oxidase but then proceeded under nitrogen as effectively as under hydrogen [13]. In fact, in the latter case, the hydrogenase assay (benzyl-viologen reduction) took place under hydrogen and it can be inferred that, when the enzyme was not already reduced by dithion-

ite, its reduction occurred during the assay itself. It is also likely that the redox potential requirement is not the same for the hydrogen uptake assay and for the exchange reaction. In the case of the uptake hydrogenase from soybean nodules for instance, the unidirectionality of the enzyme was actually related to its functioning at a relatively high potential [14]. Here, the potential requirement for the exchange appears as approaching that observed for the hydrogen production. In support to this view the optimal pH of the exchange reaction is nearly the same as the one of hydrogen production even with a hydrogenase mostly devoted to hydrogen oxidation [15].

A point to be stressed lies in the role played by cytochrome c_3 which suppressed the first stage of hydrogenase activation but did not change the activation kinetics nor the exchange rate. Cytochrome c_3 is reduced by hydrogenase under hydrogen [8]. Here, the reduction occurred in the very first minutes under hydrogen to the contact of the otherwise inactive hydrogenase. Once reduced, cytochrome c_3 could donate electrons to O_2 thus accelerating the process of hydrogenase reactivation. These experiments do not restrict the role of such a specialized molecule as cytochrome c_3 [16,17] to that of a simple oxygen scavenger, more especially as the *Desulfovibrio* genus also contains both superoxide dismutase and catalase [18]. The physiological role of cytochrome c_3 is most certainly to create specific relations between molecular hydrogen, hydrogenase and other electron carriers to allow oxidative phosphorylations during the hydrogen cycling process characteristic of sulfate-reducing bacteria [2].

The apparent discrepancy between the results obtained with *D. vulgaris* Miyazaki [8,10] and *D. gigas* ([11] and this work) could be related to a different evolutionary pattern within the genus *Desulfovibrio* during which an alteration of the role and properties of cytochrome c_3 of both species could have occurred.

More experiments based on the utilization of magnetic methods such as EPR and Mössbauer will be necessary to check whether the observed reactivation process can be related to structural changes of the iron-sulfur centers of hydrogenase in a way similar to the interconversion between 3 Fe and 4 Fe centers observed for *D. gigas* ferredoxin II [19].

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