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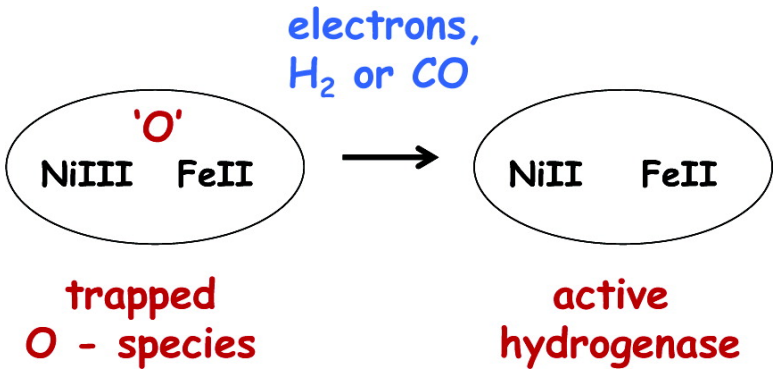

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The Mechanism of Activation of a [NiFe]-Hydrogenase by Electrons, Hydrogen, and Carbon Monoxide

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Abstract: Activation of the oxidized inactive state (termed Unready or Ni_u*) of the [NiFe]-hydrogenase from *Allochromatium vinosum* requires removal of an unidentified oxidizing entity [O], produced by partial reduction of O_2. Dynamic electrochemical kinetic studies, subjecting enzyme molecules on an electrode to sequences of potential steps and gas injections, establish the order of events in an otherwise complex sequence of reactions that involves more than one intermediate retaining [O] or its redox equivalent; fast and reversible electron transfer precedes the rate-determining step which is followed by a reaction with H_2, or the inhibitor CO, that renders the reductive activation process *irreversible*.

Introduction

Hydrogenases are found both in prokaryotes and eukaryotes[1−3] where they catalyze the interconversion between H_2 and protons, as shown in eq 1:

$$H_2 \leftrightarrow 2H^+ + 2e^- \quad (1)$$

There are three classes of hydrogenase, [FeFe]-hydrogenases (formally called [Fe]- or Fe-only hydrogenases) with two Fe atoms in the active site, [NiFe]-hydrogenases which contain a Ni and an Fe atom at the active site, and [Fe]-hydrogenases that have a single Fe atom.[2] All hydrogenases have at least one carbon monoxide as a ligand to Fe, and most have one or more cyanide ligands as well. The [FeFe]- and [NiFe]-hydrogenases also contain Fe−S clusters, which provide a relay to transport electrons in and out of the buried active site. A much-documented characteristic of the latter enzymes is that catalytic activity is lost rapidly upon exposure to O_2 in complex processes that tend to be more reversible for the [NiFe]-hydrogenases.[1] This problem has important evolutionary and physiological implications, and understanding the chemistry involved is crucial for future technological exploitation, such as designing renewable and specific catalysts for electrochemical hydrogen cycling.

The [NiFe]-hydrogenases have been studied in most detail, and the crystal structures of enzymes from different sources and in different states reveal the basic framework that is shown in Figure 1.[4,5] Various catalytic and inactive states have also been distinguished by spectroscopic techniques, notably EPR and FTIR, and investigated by theoretical methods.[6−24] At least three redox states of the active site are believed to be directly involved

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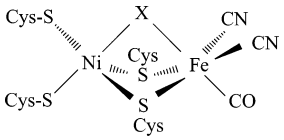


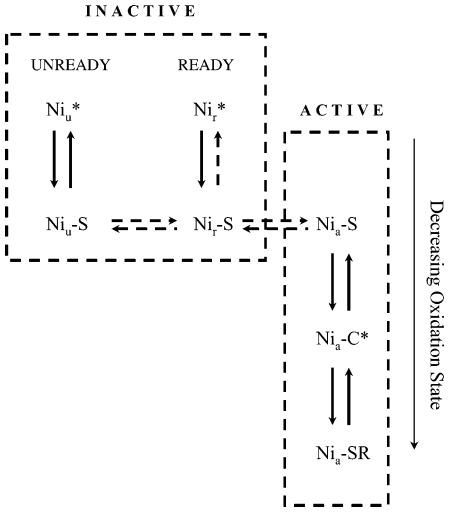
Figure 1. Minimum structure of the active site of inactive forms of [NiFe]-hydrogenase.

in catalysis; these are called Ni_a-S (or Ni−SI), Ni_a-C^*, and Ni_a-SR. However, two most oxidized states called "Unready" (Ni_u^*or Ni−A) and "Ready" (Ni_r^* or Ni−B) are inactive. As indicated in Figure 1, each of these oxidized inactive states contains a nonprotein ligand that bridges the Ni and Fe atoms, preventing catalysis, but the nature of this ligand is different cases is unclear, as are indeed so many details of the reactions involved.[3] The inhibitory ligand is believed to be oxygenic in all examples so far studied, with the exception of the enzyme from *Desulfovibrio vulgaris* (Miyazaki) for which an exogenous sulfur-containing ligand, released as H_2S upon activation, has been proposed.[5]

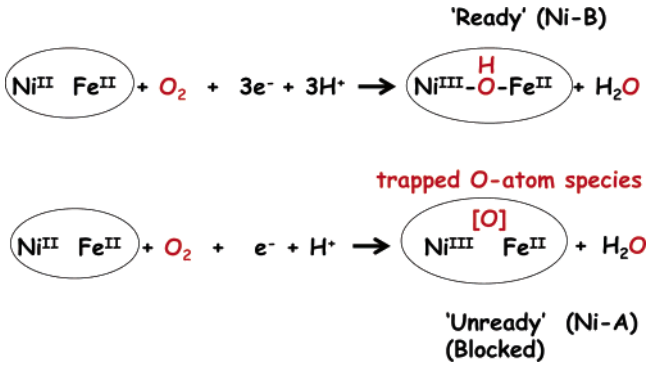
Aerobically purified enzyme from *Allochromatium vinosum*, in which the [NiFe] active site is contained in the 63 kDa subunit, contains a mixture of the Ready and Unready states, which can be activated by various reduction procedures. Allowing for variations in detail, this behavior is mirrored in other [NiFe]-hydrogenases, including the much studied enzyme from *Desulfovibrio gigas.* The Ready state is activated within seconds by low-potential electron donors and it also reacts directly with H_2.[24] However, activation of the Unready state by H_2 takes hours at room temperature, and the order of events and requirements (involvements of electron transfer, H_2, and rate-determining step) are poorly understood. In solution studies with redox mediators, both the Unready and Ready states can be converted in one-electron reactions to their respective reduced states, Ni_u-S (Ni−SU) and Ni_r-S (Ni−SI), both of which are EPR-silent and catalytically inactive but distinguished on the basis of their IR bands that arise from the CO and CN^- ligands.[22] The relationships between inactive and active states, as currently understood, are summarized in Scheme 1.

The oxidation status of the enzyme and overall availability of electrons are defined also by the oxidation levels of the Fe−S clusters, contained in the 32 kDa subunit. In the text, this is indicated by adding the shorthand notation contained in brackets (o = oxidized, r = reduced, for clusters listed in the order proximal, medial, distal). Thus Ni_r*(oro) refers to the Ready state in which the medial [3Fe−4S] cluster is reduced and the proximal and distal [4Fe−4S] clusters are oxidized.

Scheme 1. Current General Scheme of Reactions Showing the Relationship between Active and Inactive States of [NiFe]-Hydrogenase



Scheme 2. Reactions of Active [NiFe]-Hydrogenase with O_2 Depend on the Number of Electrons Available, Provided Either by H_2 Turnover Activity or by the Electrode



Recent studies have provided important new insight into the *specific* chemical differences between Ready and Unready states that account for their contrasting rates of activation. To summarize, reports by George et al.[23] and Lamle et al.[25] support a new proposal that whereas the Ready state contains a bridging OH^- ligand that is derived either from solvent water or complete four-electron reduction of O_2, the Unready state contains a *partially* reduced O_2 species [O] that is trapped in the active site. This would not only account for the greater difficulty of activating Unready but also explain why it is difficult (or even impossible) to achieve an intrinsic equilibrium between Unready and Ready states. These two pathways, each yielding a Ni(III) product with nonmagnetic (two- or four-electron) products of O_2 reduction, are represented in Scheme 2. One option is that the [O] species is trapped as a sulfoxide (>S═O) or sulfenic acid (−SOH) functionality located on one of the cysteine residues that coordinate the Ni. Interestingly, the process by which this occurs would resemble the action of a monooxygenase. Studies with simple Ni thiolate compounds have indeed shown that S-oxidation products are formed upon reaction with O_2.[26] The sulfenic acid functionality is known to be highly

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reactive, but examples of CysS−OH are now reported for several enzymes, including nitrile dehydratase.[27] The other option is a peroxide species that becomes trapped and unable to leave the active site unless released by reduction or rearrangement.[28] Obviously, *both* of these situations may occur together, with one species in equilibrium with the other.

Studies of *A. vinosum* [NiFe]-hydrogenase by protein film voltammetry, in which the enzyme is adsorbed on a pyrolytic graphite "edge" (PGE) electrode, show that the active site oxidizes H_2 at a remarkable rate, comparable to that of a Pt catalyst.[29,30] Many fundamental properties have been examined by exploiting this activity to make sensitive voltammetric measurements.[25,31−35] Protein film voltammetry has unique attributes for studying redox catalysts as complicated as hydrogenases, a particularly relevant one being that convoluted electron-transfer and chemical processes are easily observed and resolved in both potential and time domains.[36] Importantly, the catalytic activity (turnover rate) of a minuscule sample of enzyme is measured *directly* as current. In this respect, since high activity provides excellent signal-to-noise, we have a powerful tool with which to measure the kinetics of processes initiated by a potential-step perturbation or injection of gas, while the enzyme is held under strict potential control.

A paper in 2003 focused on the *anaerobic* electro-oxidation reaction and reductive reactivation of the product, which was assigned as the Ready state.[33] Léger and co-workers subsequently studied the kinetics of inactivation, by O_2, of the [NiFe]-hydrogenase from *Desulfovibrio fructosovorans*, over a range of pH and electrode potential, concluding that the rate was independent of these variables.[35] Recently, we showed[25] that Ready and Unready products are distinguished clearly by their kinetics of reactivation that are observed upon applying a reductive potential step. We were able to exploit this technique to establish that although both Ready and Unready states are produced rapidly when active enzyme is exposed to O_2, the *ratio* of Unready to Ready increases steadily and reproducibly as the enzyme is deprived of electrons at the time of exposure, leading to the proposal shown in Scheme 2 in which Unready is an "oxygenated" form of the enzyme. In this paper, we address the mechanism of activation of the Unready state, a complex process that challenges microbial life and future technological applications. Aware that voltammetric methods yield kinetics and energetics rather than structural data, our aims were modest; yet such a study was crucial for establishing the temporal and energetic order of events in what is otherwise a surprisingly complex sequence. We have also been able to illuminate the significant proposal, first made in the 1980s,[37] that CO is not only an inhibitor but also an activator of [NiFe]-hydrogenase.

Materials and Methods

The [NiFe]-hydrogenase from *A. vinosum* (*Av*) was prepared as described previously.[8] All experiments were carried out anaerobically in a glovebox (M. Braun) under N_2 (O_2 < 2 ppm). The all-glass electrochemical cell was equipped with an "o" ring gasket, which was fitted around the electrode rotator to seal the internal headspace of the electrochemical cell from the glovebox atmosphere.[33] This design allowed gases to be introduced quickly and reliably at a constant pressure through inlet and outlet sidearms, and less than 5 min was required to achieve complete equilibration with the cell solution when the electrode was rotating at high speed. The rotating disk pyrolytic graphite edge (PGE) electrode,[38] driven by a EG&G M636 electrode rotator, was polished with an aqueous alumina slurry (1 μm Al_2O_3, Buehler) and sonicated thoroughly before each experiment. The counter electrode was a piece of platinum wire, and the saturated calomel reference electrode (SCE) was situated in a Luggin sidearm filled with 0.1 M NaCl. The main cell compartment was jacketed and thermostated at the required temperature, while the reference electrode sidearm was well separated and kept at a constant temperature (a nonisothermal configuration). The reference potential was corrected with respect to the standard hydrogen electrode (SHE) by using $E_{SHE} = E_{SCE} + 242$ mV at 25 °C.[39] All values were adjusted to conform to the SHE scale. Electrochemical experiments were performed with an Autolab PGSTAT 20 or PGSTAT 30 electrochemical analyzer (Eco Chemie, The Netherlands) controlled by GPES software (Eco Chemie, The Netherlands). Cyclic voltammetry without enzyme showed no currents due to H_2 oxidation or proton reduction over the range of at least 400 to −650 mV. For the potential-step experiments, the current was sampled every second following the step (duration ≪ 1 s).

A mixed buffer system was used in all experiments. This consisted of 15 mM in each of sodium acetate, MES (2-[*N*′-morpholino]-ethanesulfonic acid), HEPES (*N*′-[2-hydroxyethyl]piperazine-*N*′-2-ethane-sulfonic acid), TAPS (*N*′-tris[hydroxymethyl]methyl-3-amino-propanesulfonic acid), and CHES (2-[*N*′-cyclohexylamino]ethanesulfonic acid), each purchased from Sigma, with 0.1 M NaCl as supporting electrolyte. All solutions were prepared using purified water (Millipore 18 MΩ cm) and titrated with NaOH or HCl to the desired pH at the experimental temperature. A co-adsorbate, polymyxin B sulfate (from a stock solution), was added to all cell solutions (final concentrations 200 μg mL^{-1}) to stabilize the protein film. Concentrations of O_2 were estimated assuming that the concentration in the stock solution injected into the cell was that expected for equilibrium with 1 bar at room temperature (i.e., 1 mM at 45 °C).[40]

Each film of *A. vinosum* hydrogenase on the electrode was formed as described previously.[29] This included holding the electrode with the enzyme film at −550 mV under H_2 at 45 °C for 30 min to ensure the whole sample was in the active form. At this stage, when H_2 is introduced into the cell, the electrode exhibits diffusion-controlled H_2 oxidation.[29−33] This causes a problem when trying to monitor subtle changes in enzyme activity (diffusion control masks the details of the catalytic properties of the enzyme). Therefore, the electrode was "polished" gently with damp cotton wool to remove some of the enzyme from the surface. This produces a "depleted" film for which the catalytic current is independent of electrode rotation rate within a large range (1500−2500 rpm). The depleted film electrode was then transferred to

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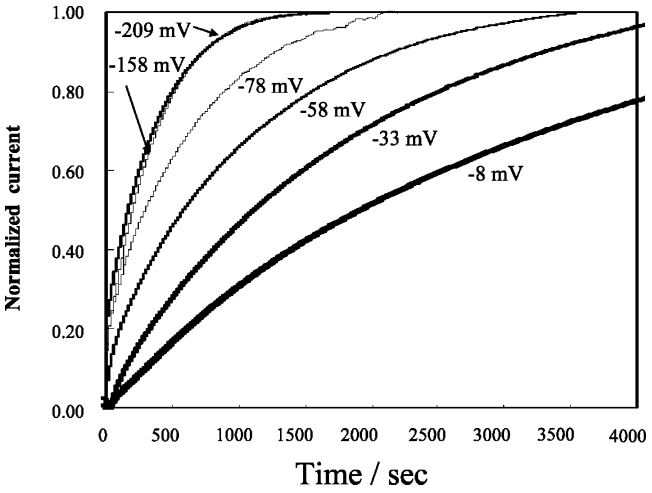


Figure 2. Activation of the Unready state of [NiFe]-hydrogenase under H_2 (1 bar) initiated by stepping the electrode potential down to various values. After incubating under N_2 then injecting O_2 at 242 mV, H_2 was used to flush out the remaining O_2 for 600 s before the potential was stepped to various reducing potentials (−8, −33, −58, −78, and −158 mV), and the increase in activation was monitored. Other experimental conditions include the following: pH 6.0, 45 °C, and electrode rotation rate = 2500 rpm.

the electrochemical cell containing pristine buffer−electrolyte, free of enzyme, whereupon numerous sequential experiments could be undertaken over several hours with the same enzyme sample.

The temperature of 45 °C used for most experiments allows the activation of Unready to be monitored to completion within a reasonable time scale (ca. <30 min); otherwise, at room temperature, several hours were required. Most experiments were carried out at pH 6.0 to minimize anaerobic inactivation (formation of the Ready state is slower at low pH), and the enzyme film on the electrode is more stable than at higher pH.[29,33]

Results

In a recent paper,[25] we showed that the rate of activation of the Unready state is independent of electrode potential in the region from −108 to −308 mV, that is, well below a value called E_{switch}, measurable by cyclic voltammetry at slow scan rates, which defines the potential at which the enzyme undergoes reductive reactivation. Since activation requires reducing conditions, this result suggested that a limiting rate is reached once the potential is sufficiently negative to populate the centers involved and initiate activation. We therefore conducted further experiments to help elucidate the nature of this process. Figure 2 shows the results of experiments carried out, as before, with a depleted film of [NiFe]-hydrogenase on a rotating disk PGE electrode, except that activation is initiated by stepping to less-negative electrode potentials. In all cases, the sample of hydrogenase on the electrode had been subjected previously to a sequence of potential steps, gas exchanges, and O_2 injection known to generate >80% of enzyme in the Unready state.[25]

Thus, after forming a film of enzyme on the electrode, as described in Materials and Methods, the potential was held at −558 mV for 1200 s under N_2 before being stepped to 242 mV, at which point 0.1 mL of O_2-saturated buffer was injected. This results in immediate loss of activity. The headspace was then flushed with H_2 for 600 s to remove residual O_2 before the potential was stepped back down to more negative values to initiate reactivation. As described previously, at potentials more negative than −100 mV, approximately 15% of the activity

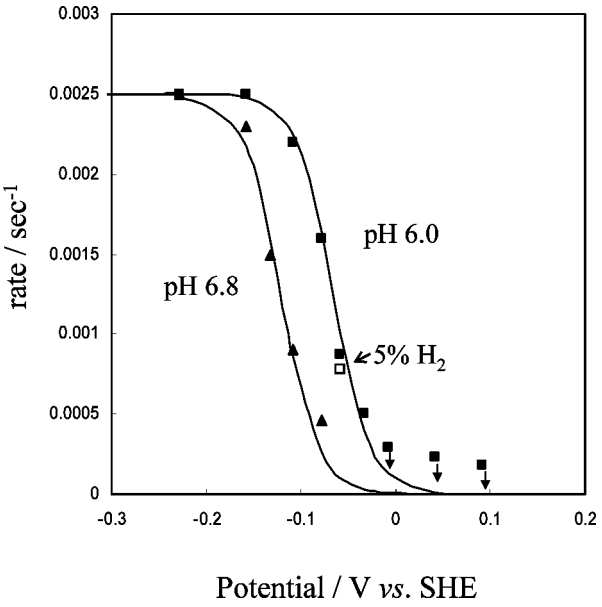


Figure 3. Dependence of the rate of activation of Unready [NiFe]-hydrogenase on electrode potential, measured at 45 °C. Data derived from experiments shown in Figure 2 and others carried out at pH 6.8. Apart from the datum point indicated by an arrow (open square), all experiments were carried out under 1 bar H_2. Lines represent fits using eq 6, explained in the Discussion.

is regained immediately in a fast phase; this is followed by a slow exponential phase lasting many minutes, the rate constant of which is independent of potential. These two phases correspond to activation of Ready and Unready states, respectively.[25] Stepping to less negative potentials also produces exponential activation traces, but with rate constants that decrease dramatically as the electrode potential is increased above −100 mV. Only at the more negative potentials (−79, −158, and −209 mV) is the initial fast phase due to activation of Ready clearly discernible, which is as expected since the rate of activation of Ready decreases very rapidly as the potential is increased.[33] Excellent first-order kinetics were observed over 3−4 half-lives provided the potential was sufficiently negative to reach completion within the normal duration of the experiments (approximately 1 h). At the higher potentials, the activation of Unready is so slow that the end point is not reached within 2 h, during which time anaerobic inactivation and natural film loss have also occurred. These data were analyzed using the Guggenheim procedure;[41] nevertheless, the rates are probably an over-estimate.

Figure 3 shows how the rate constants vary as a function of potential, and data are given in Table 1. In accordance with our previous study, the sigmoidal-type dependence reaches a limiting rate of 0.0025 s^{-1} below −100 mV; the downward arrows on the data points at high potential signify the direction of uncertainties due to the very slow rates. The line shown is the best fit to the model that is derived later. Included in the plot is the result of an experiment carried out under much lower H_2 partial pressure (5% H_2 in N_2), which clearly lies close to the same line. Analogous experiments to those shown in Figure 2 were carried out at pH 6.8, and the results are plotted separately on the same graph. Here, due to competing anaerobic inactivation that is more rapid at higher pH, we did not obtain

(41) Moore, J. W.; Pearson, R. G. *Kinetics and Mechanism*, 3rd ed.; Wiley: New York, 1981.

Table 1. Rates of Activation of the Unready State of *Allochromatium vinosum* [NiFe]-Hydrogenase as a Function of Potential (pH 6.0 or 6.8, $T = 45$ °C, 1 bar H_2, unless otherwise stated)

pH 6.0		pH 6.8	
potential/ mV vs SHE	$10^3\times$ rate constant/s^{-1}	potential/ mV vs SHE	$10^3\times$ rate constant/s^{-1}
92	0.18	−78	0.46
42	0.23	−108	0.86
−8	0.29	−133	1.50
−33	0.50	−158	2.30
−58*	0.75[a]	−228	2.49
−58	0.87	−250	2.50
−78	1.64		
−108	2.24		
−158	2.50		

[a] Rate measured under 5% H_2 in N_2.

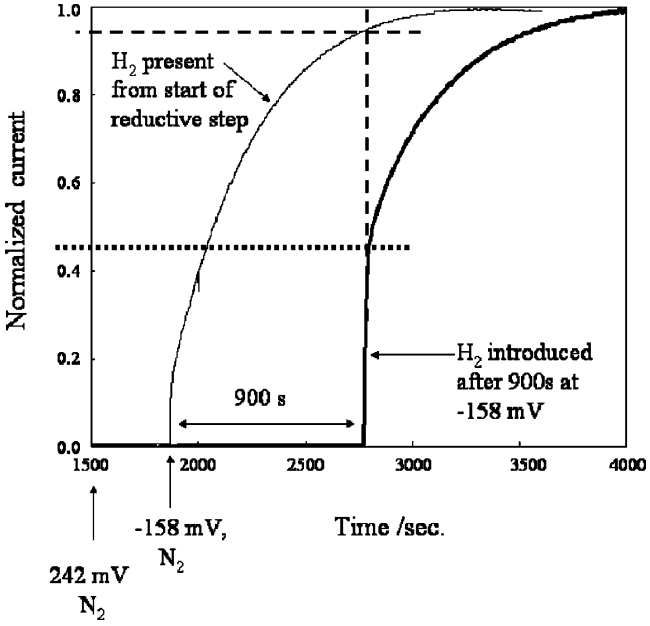


Figure 4. Experiments demonstrating the effect of H_2 on activation of [NiFe]-hydrogenase. The thin line shows the experiment in which an active film of *Allochromatium vinosum* [NiFe]-hydrogenase was first converted to >80% Unready by incubating under 1 bar N_2, then stepping to 242 mV and injecting 0.1 mL of O_2-saturated buffer. H_2 was then passed through the cell for 600 s to flush out the remaining O_2 before the potential was stepped to −158 mV and the current response recorded (thin black line). The broad line shows an experiment carried out as before, except that after inactivation to generate >80% Unready, the film was held for 600 s at 242 mV under N_2 and then at −158 mV for 900 s under 1 bar N_2 before 1 bar H_2 was introduced into the cell. Other experimental conditions include the following: 45 °C, pH 6.0, and electrode rotation rate = 2500 rpm.

data for the higher potentials at which reactivation is very slow. In comparing the two sets of data, two aspects are clear; first, the limiting rate at negative potential is unchanged between pH 6.0 and 6.8, thus confirming our earlier report; second, as the pH is increased, the sigmoidal wave midpoint potential shifts from −65 to −120 mV. The fits to these data will be described later.

Figure 4 shows potential-step chronoamperometry experiments that provide an important clue as to the role of H_2 in the activation of the Unready state. First, in the typical manner (see Figure 2), the course of reductive activation was monitored in the presence of 1 bar H_2 (thin black line). The trace shows that greater than 90% of the enzyme has become active after 15

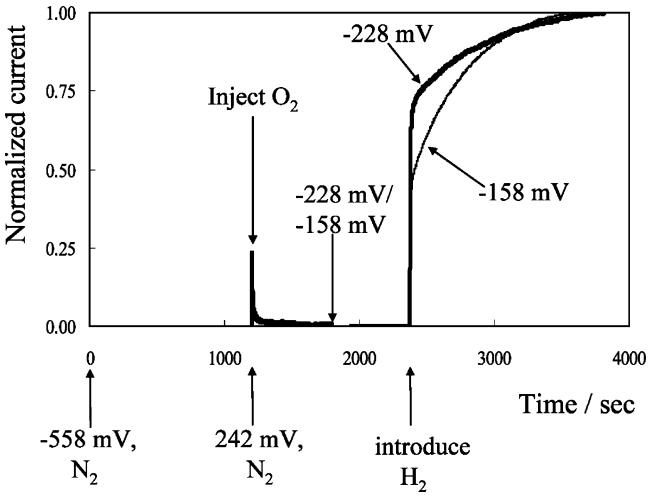


Figure 5. Experiments showing the effect of electrode potential when preincubating [NiFe]-hydrogenase under N_2 (1 bar) prior to exchanging with H_2 (1 bar). In each case, an active film of hydrogenase was first converted to >80% Unready state by incubating under 1 bar N_2 then stepping the potential to 242 mV and injecting 0.1 mL of O_2-saturated buffer. N_2 was used to flush out the remaining O_2 for 600 s before the potential was stepped to either −158 or −228 mV. After 600 s, 1 bar H_2 was introduced into the cell, and the current responses were recorded. Other experimental details include the following: pH 6.0, 45 °C, and electrode rotation rate = 2500 rpm.

min at −158 mV. Second, the experiment was repeated using the same sequence of operations, except that after O_2 injection, the enzyme was incubated at −158 mV under N_2 instead of H_2, then at a precise point in time (15 min), the headgas was changed to H_2 to measure how much activity had been recovered. (Control experiments under the same conditions showed that gas exchange is complete within 5 min.) The resulting trace shows clearly that a much smaller fraction (approximately 45%) of enzyme has been activated when poised for 15 min under N_2 compared to 15 min under H_2.

This discovery was explored further in several ways. First, reductive activation under N_2 was allowed to proceed for different times before changing the headgas to H_2. For each run, the fraction of active enzyme was estimated by extrapolating back to the time of H_2 exchange and correcting for the amount of enzyme expected to be produced in the Ready state (approximately 15%) based on experiments carried out under the same conditions of O_2 injection. The same experiments were then repeated but using different potentials to activate the enzyme. In most cases, the same enzyme film was used to perform several successive cycles of measurements. Figure 5 shows the results of two experiments, one where the enzyme was reactivated under N_2 for 10 min at −228 mV before H_2 was introduced, and an identical experiment in which the electrode potential was held at −158 mV.

The results show that at −228 mV, a greater proportion of the enzyme has been converted to an "active" form (i.e., shown to be active immediately upon addition of H_2) after 10 min (70% compared to 40%). In either case, the rate of the slow phase is independent of potential and close to the value of 0.0025 s^{-1} (see Figure 3) observed as the limiting rate for activation under H_2 at potentials below −100 mV. The results of many similar experiments are summarized in Figure 6, where the potential of reactivation and duration of the poise under N_2 has been varied. The fraction of enzyme estimated to be "instantly"

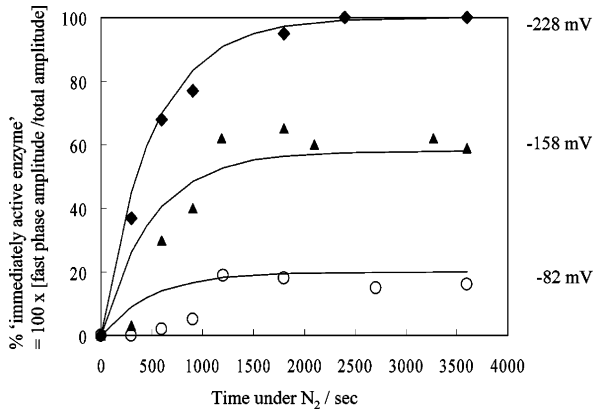


Figure 6. Graphs showing the fractions of enzymes that are "immediately" activated upon addition of H_2 after equilibrating at different potentials and times under N_2 (1 bar). All experiments were carried out under conditions described in the legend to Figure 5.

activated upon exchanging N_2 for H_2 has been plotted against the time spent under N_2. In all cases, this fraction reaches a limiting value after about 30 min. The limiting value depends on the electrode potential; thus at −228 mV, greater than 90% of the enzyme is activated, whereas at −82 mV, the amount is less than 25%.

The curves do not represent fits to any expression, and clearly, the data at short times and high potentials show considerable scatter. Despite this, the important inference is that in the absence of H_2, an *equilibrium* is established between the electrode and the active site of hydrogenase. As discussed later, this is a new and crucial observation for understanding the activation process in which a partially reduced [O] species is trapped by the enzyme. The rate at which the equilibrium state is reached is approximately the same (half-life of 5−10 min) in all cases, noting that the error margins in these experiments are significantly larger than those for the direct measurements of the progress of activation under H_2. We conducted an experiment, shown in Figure 7, to confirm the reversibility of this process. Incubation under N_2 at −228 mV was carried out for 25 min (expected to provide >85% rapid activation upon exchange with H_2), then instead of exchanging with H_2 at this point (a trace showing a result obtained in this case is shown for comparison, panel A), the potential was stepped back up to −78 mV and held at this value for 25 min, before exchanging H_2 into the headspace (panel B). The much lower fraction of enzyme able to activate immediately (20% compared to 85%) confirmed the reversibility of the process and showed unequivocally that an equilibrium is established when H_2 is absent. It is important to stress that, in all cases, the total amount of activity recovered was always more than 80−90% of that measured before the cycle of operations was started.

On the basis that the rate-determining step produces, reversibly, a precursor rather than (irreversibly) a fully active species, we carried out an experiment to determine how important is the rate of reaction of this precursor with H_2. The enzyme was held at −558 mV under N_2 at 45 °C before the potential was stepped to 242 mV and 0.1 mL of O_2-saturated buffer was injected into the cell. After flushing with N_2 for 10 min, the potential was stepped down to −228 mV under N_2 and held there for 55 min before the temperature was cooled to 10 °C (5 min) and H_2 introduced. Despite the much lower temperature, immediate activation was observed upon addition of H_2,

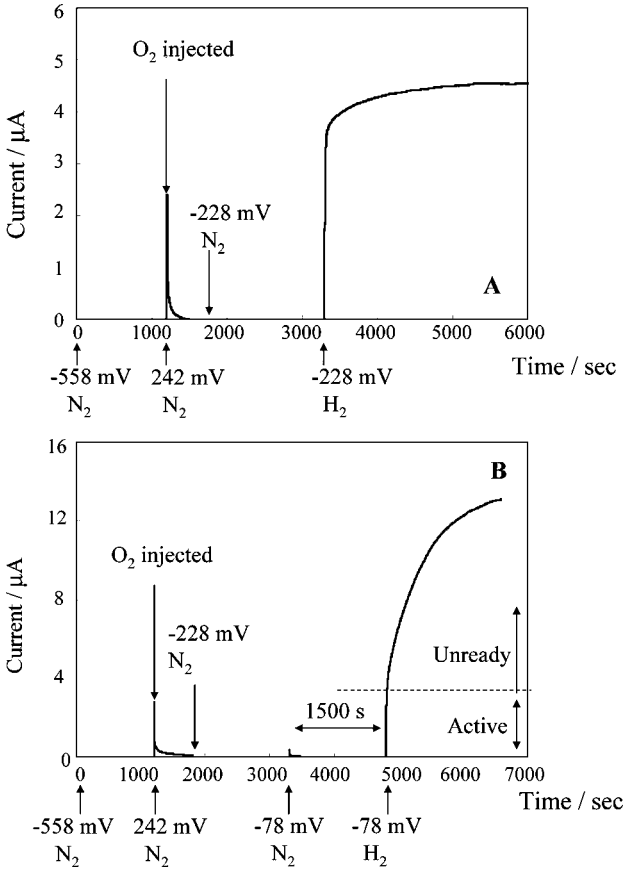


Figure 7. Experiment demonstrating that formation of the state P that activates "instantly" upon exchanging with H_2 (1 bar) can be titrated back to the oxidized Unready state. (A) Experiment carried out as described in Figure 5; enzyme reactivated for 25 min under N_2 at a reactivation potential of −228 mV before H_2 was introduced. (B) Back-titration experiment carried out as described for A except that after 25 min at −228 mV under N_2, the potential was increased to −78 mV for 25 min before H_2 was introduced. Other experimental details include the following: pH 5.9, 45 °C, and an electrode rotation rate = 2500 rpm.

increasing to a maximum amplitude at a rate similar to that observed in experiments carried out at 45 °C.

We reported recently that CO appears to activate Unready hydrogenase that has otherwise not encountered H_2. This was now investigated further to see if we could establish how fast this process is and whether CO could activate hydrogenase at relatively high potential. Figure 8A shows an experiment in which CO has been introduced during the activation period prior to exchanging H_2 into the headspace, the potential step sequence otherwise being the same as described for Figure 4. In this case, the activation period of 25 min at −158 mV comprised 20 min under CO followed by 5 min of flushing with N_2 (to remove free CO from solution) before H_2 was introduced. Upon introducing H_2, the activity rose rapidly to a maximum value after approximately 180 s without any observable fast and slow phases. The thin black line in Figure 8A shows an experiment in which no CO was introduced, and the entire activation at −158 mV was under N_2. Further experiments were carried out using an identical potential-step sequence but varying the length of time the enzyme was exposed to CO. Obviously, the shortest time possible was limited by the rate of gas exchange. Even so, after only 10 min under CO at 45 °C, more than 90% of the enzyme is activated directly upon addition of H_2. A further experiment was carried out in which CO was allowed to react

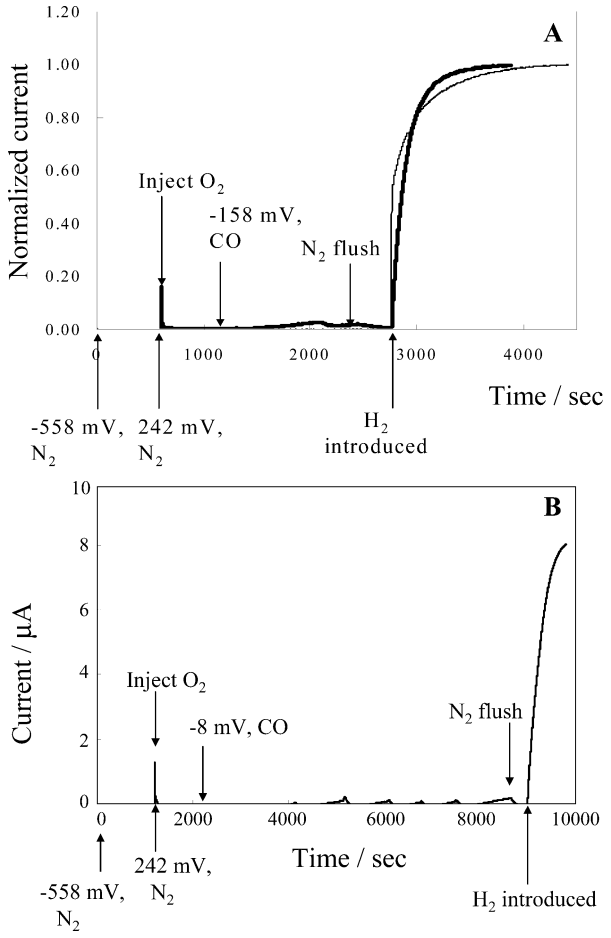


Figure 8. Experiment to determine the effect of introducing CO during the period that Unready enzyme is held at reducing potentials under N_2 (1 bar). (A) Experiment in which the effect of CO is compared with that of N_2. Thick black line shows an experiment where (after inactivation) the enzyme was reactivated at −158 mV under CO for 20 min before N_2 was used to flush (5 min) the cell of free CO before H_2 was introduced. Thin black line shows an experiment with no CO present, where the reactivation occurred under N_2 for the full 25 min before H_2 was introduced. (B) Experiment demonstrating that CO results in activation of hydrogenase at a relatively high potential. Experimental conditions were the same as described in A (thick black line), except that the reactivation occurred at a potential of −8 mV for nearly 2 h, then N_2 was used to flush the cell for 5 min before H_2 was introduced. Other experimental details include the following: pH 5.9, 45 °C, and an electrode rotation rate = 2500 rpm.

for more than 2 h with the electrode potential held at −8 mV [at which no activation should occur under N_2 (Figure 6), and activation under H_2 should be very slow (as shown in Figure 2)]. The CO was then removed by exchanging with N_2 while the electrode potential was maintained at −8 mV for a further 5 min. When H_2 was added, at least 70% of the enzyme was activated within 5 min.

Discussion

We separate this discussion into *qualitative* and *analytical* sections. Four definitive, qualitative conclusions can be drawn:

(1) The rate of reductive activation of enzyme in the Unready state under 1 bar H_2 (in which electrons are provided directly and rapidly at an electrode) exhibits a steep potential profile; at pH 6.0, the rate increases from almost zero above 0 mV to a maximum rate just below −100 mV. The limiting rate of 0.0025 s^{-1} at 45 °C, which (using the activation energy of 88 kJ mol^{-1} [25]) extrapolates to 0.00018 s^{-1} ($t_{1/2}$ = ~1 h) at 20 °C, compares with values measured by other methods for the enzyme from *Desulfovibrio gigas*.[14,16] At pH 6.8, the curve shifts to a more negative potential (thus it becomes thermodynamically more difficult to reactivate the enzyme), but the limiting rate reached at low potential is unchanged.

(2) In the absence of H_2, electrons alone result in a slowly established *reversible* equilibrium between Unready and an intermediate state that is still not active, but rather a precursor of the active enzyme. The observation that application of an oxidizing potential restores the Unready state (Ni_u* or Ni−A) rather than producing the Ready state (Ni_r* or Ni−B) shows that the intermediate species cannot be a specific reduced form of the Ready state (Ni−SI). Interestingly, the equilibrium potential for this interconversion is approximately 80 mV more reducing than that observed from the kinetic profile when experiments are made under H_2.

(3) As a consequence of (2), both H_2 *and* electrons, separately, are required to activate the Unready state.

(4) Carbon monoxide is an activator in place of H_2.

It is well-known that the activation of Unready [NiFe]-hydrogenase is a slow process at room temperature, often taking many hours, whereas the activation of Ready, which involves removal of a bound hydroxo species, is fast.[24,33] In a recent paper,[25] we studied the formation of Unready under strict electrochemical control, and in addition to determining the conditions for its formation by reaction with O_2, we established that in the *presence* of H_2, reductive activation at 45 °C proceeded with clean, first-order kinetics. At sufficiently low potentials, that is, below −100 mV, the rate constant is independent of electrochemical driving force and pH. Our studies now define the full potential dependence of activation of the Unready state over a wide range; they reveal also that an equilibrium is established in the absence of H_2, and consequently that full activation depends on the presence of H_2 (or indeed CO).

Recent studies have shown that the Unready state does not react directly with H_2, leading to the proposal that the well-known activation of Unready by H_2 is the result of reaction of H_2 with small amounts of enzyme in the Ready state, which then becomes catalytically active and provides a source of electrons.[24] One object of the present study was therefore to elucidate whether electrons *alone* are able to activate Unready. By allowing the enzyme to take up electrons under a N_2 atmosphere, we determined that this does not produce an active enzyme; instead, an equilibrium is established in which the "end product" can be back-titrated to Unready simply by increasing the potential. This end product is thus identified as a crucial intermediate that converts rapidly to active enzyme *if* H_2 is present. Without the use of protein film voltammetry, which can resolve electron and chemical events in the time and potential dimensions, it is unlikely that this aspect would be revealed; previous studies of hydrogenase activation have usually been carried out with H_2 present or (if H_2 was absent) the medium contained dithionite and/or other low-potential electron donors that will generate H_2.[7,8,14−16,42,43]

We can now analyze the results in more detail. From EPR titrations, it is known that Unready is reduced to Ni_u−S in a rapid and reversible titration that involves one electron and one proton.[7,8] This occurs with a reduction potential of −80 mV at

pH 6. The [3Fe−4S] cluster has a slightly higher potential; we have determined this as −30 mV.[29]

The general reaction scheme that starts with a rapid electrochemical pre-equilibrium (reduction of O to R) followed by a slow reversible step to give precursor P and then a final fast irreversible reaction (with H_2) is expressed simply as follows (eq 2)

$$O \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P \xrightarrow{k_3} \quad (2)$$

In more descriptive terms, this can be presented as eq 2A

$$O \underset{\text{(fast)}}{\overset{\text{pre-equilibrium}}{\rightleftharpoons}} R \underset{\text{(slow)}}{\overset{\text{reversible}}{\rightleftharpoons}} P \xrightarrow[\text{commitment}]{} \text{active enzyme} \quad (2A)$$

Starting from the rate equation[44] for this sequence, which is eq 3

$$\text{rate} = \frac{k_1 k_2 k_3}{k_{-1}k_{-2} + k_3 k_{-1} + k_2 k_3 + k_1 k_{-2} + k_1 k_3 + k_1 k_2} \quad (3)$$

we can write, since $K_1 = k_1/k_{-1}$

$$\text{rate} = \frac{K_1 k_2 k_3}{K_1(k_{-2} + k_3 k_2) + k_{-2} + k_3 + (k_2 k_3/k_{-1})} \quad (4)$$

Then since K_1 is an electrochemical equilibrium

$$K_1 = \exp\{nF(E^0 - E)/RT\} \quad (5)$$

where E^0 is the reduction potential of the center(s) responsible, E is the electrode potential, n is the apparent number of electrons transferred, F is the faraday constant, R is the gas constant, and T is the absolute temperature, we can write eq 6, which relates the rate of activation to the applied electrode potential.

$$\text{rate} = \frac{k_2 k_3 \exp\{nF(E^0 - E)/RT\}}{k_{-2} + k_3 + (k_2 k_3/k_{-1}) + \exp\{nF(E^0 - E)/RT\}(k_{-2} + k_3 + k_2)} \quad (6)$$

In deriving this equation, we have made the simplifications $k_1 = k_1'[H^+]$ and $k_3 = k_3'[H_2]$, where k_1' and k_3' are second-order rate constants, as required.

Figure 3 includes the best fit to eq 6. This yields k_2 as the rate-determining step with a value of 0.0025 s^{-1}, as expected. At high potential, the rate should decrease to zero; the experimental data lie above the line, but we believe that this arises from over-estimation of rate constants due to the reaction being so slow that (a) an infinity value was not obtainable, and (b) there is competing film loss and anaerobic inactivation. The fit is insensitive to k_3 as long as this is much larger than k_{-2}, again, as expected. As k_{-1} becomes very small (i.e., <0.001 s^{-1}), the position of E^0 shifts to more negative values. This is because k_{-1} (and k_1) relates to the exchange rate constant k_0 (via the Butler−Volmer model for electrochemical kinetics)[39] so it may be considered to reflect the interfacial electron-transfer kinetics. Consequently, a higher electrochemical driving force is required to transfer an electron into the enzyme. On the basis of the time scale of the regeneration of Unready, k_{-2} has been estimated at approximately 0.001 s^{-1} (but with large errors). Decreasing this value further has little effect on the appearance of the slope. The reduction potential E^0 is in good agreement with the value E_{switch} determined from the inflection point of the rise in activity observed in voltammograms (scanned in the negative direction) measured at very slow scan rates.[25] The fit and even the basic shape are sensitive to n, and although a reasonable fit was obtained with $n = 1.0$, the best fit was obtained with $n = 1.4$ (see below).

From our previous study, the Unready state contains an oxidizing entity that is the product of partial reduction of O_2. The obvious options are a trapped O atom, which may reside on one of the cysteine S atoms, or a peroxide. We now try and fit these data into a model.

The rapid pre-equilibrium can be identified with the one-electron reduction of Unready, which is well established from EPR titrations to be fully reversible.[8] The tendency for n to exceed 1.0 is at first sight unexpected, yet it has an interesting explanation since if only one electron were to be transferred to the enzyme in its Unready state, it would reside predominantly on the [3Fe−4S] cluster. The slightly more negative reduction potential of the Unready state Ni(III) means that Ni_u*(oro) is more stable than Ni_u(ooo), so that on average, more than one electron will be required to ensure that an electron goes to the Ni. At pH 6.8, the potential has shifted in the negative direction by approximately 55 mV, indicative of the simultaneous coupled transfer of one proton.

When measured under N_2, the reaction proceeds as far as species P in a slow process that has similar kinetics to that of activation under H_2. When H_2 is then added to the cell after holding the potential below −200 mV for 30 min, the fact that almost 100% of the expected enzyme population is observed to be immediately active requires that nearly all the sample has *traversed* the rate-determining step, that is, that species P is present and is predominant over R. The equilibrium situation is clearly distinguished from the steady-state situation that applies under H_2, where species P is removed as soon as it is formed (so it does not need to be stabilized). In simple terms, the steady-state kinetics (under H_2) reports on the kinetic pre-equilibrium O ↔ R, whereas the equilibrium experiment (under N_2) measures O ↔ P. This is an informative dilemma. If the transformation of R to P under N_2 is the same process as we have modeled successfully for the kinetics under H_2, the equilibrium O ↔ R ought to be coupled to a favorable conversion of R to P; yet the equilibrium reduction potential measured under N_2 is actually more negative (overall, it is less spontaneous) than that measured under H_2. Put another way, the potential required to *accumulate* species P (under N_2) is lower than that expected from the steady-state model (under H_2) in which P is a transient species. The results in Figure 6 show that a potential of −82 mV does not produce a significant amount of any species that is "ready to go" once H_2 is added. This discrepancy is outside the limits of error, even taking into consideration the time taken for H_2 to exchange with N_2 and the large scatter at low amplitudes.

We list the following possibilities, all of which may apply.

(1) H_2 influences the reduction potential for the fast pre-equilibrium O ↔ R.

(42) Lissolo, T.; Pulvin, S.; Thomas, D. *J. Biol. Chem.* **1984**, *259*, 11725−11729.
(43) Mege, R.-M.; Bourdillon, C. *J. Biol. Chem.* **1985**, *260*, 14701−14706.
(44) Purich, D. L.; Allison, R. D. *Handbook of Biochemical Kinetics*; Academic Press: San Diego, CA, 2000.

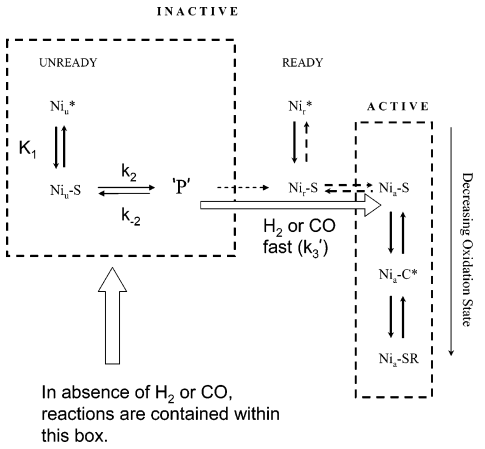
(2) H_2 influences the equilibrium position for the process R ↔ P.

(3) The transformation R ↔ P involves a further electrochemically driven redox reaction (such as reduction of the [O] atom species to water).

On the basis of the fact that studies carried out with a lower H_2 partial pressure (5% in N_2) showed only a small decrease in activation rate as obtained at 1 bar H_2, (equivalent to less than 20 mV negative shift in potential), we believe that the first possibility is unlikely to account for the discrepancy. At first glance, the second possibility is likely if H_2 binds selectively to species P, the precursor to active enzyme; the lower reduction potential for O ↔ P relative to O ↔ R (effectively a destabilization of P) is indeed observed when H_2 is absent, but this is still not fully consistent with the observation that P is formed *after* the rate-determining step *and* in a potential-dependent equilibrium. The third possibility can be discounted because [O] atom species are always derived from reduction of O_2 and it is difficult to see, energetically, how R and O could be re-formed from P if this contained, for example, an oxide or hydroxide. Put another way, since an active-site peroxide, or indeed any other product of partial reduction of O_2, is unlikely to be produced from solvent aqua species under such reducing conditions, we conclude that the precursor P which produces active enzyme once H_2 is added has the following characteristics: (a) it *must still contain* the oxidizing entity; (b) it must contain this entity in a form that can be displaced by H_2 (indeed, until H_2 is introduced, species "P" can be reoxidized to give back the Unready state). It follows naturally that none of these species contains an unusual exogenous ligand (such as a sulfur-containing entity[5]) that is ultimately released to solvent since multiple inactivation/reactivation cycles could be carried out on the same enzyme sample (≪picomole) in contact with clean electrolyte without observing any change in behavior.

Notwithstanding this unresolved dilemma, our results now provide compelling evidence for the presence of more than one [O]-containing species that may be difficult to distinguish by spectroscopic methods. For example, both R and P, which interconvert slowly, are probably Ni(II) species, and thus EPR-silent. One could speculate that one form contains a modified cysteine, as S═O or SOH (R?), and the other contains a peroxide (P?). However, conversion of a peroxide to a S−O species ought not to be reversible because S−O or S═O bonds are stronger than those of O−O.[45] Consider the need for H_2 to complete the activation process and the evidence that CO can serve in place of H_2; assuming CO is not enzymatically oxidized, this suggests that the final step is a displacement, possibly from a terminal position on the Ni, at which CO is likely to bind.[13] Consider also that the ultimate rate limitation, k_2, is independent of pH and solvent H versus D, and that the activation parameters, $\Delta H^{\ddagger} = 84.2 \pm 0.7$ kJ mol^{-1} and $\Delta S^{\ddagger} = -28 + 5$ J K^{-1} mol^{-1}, suggest[25] an ordered transition state with no cleavage of strong bonds. On the basis of these separate items of kinetic evidence, it is more likely that the [O]-containing species is a peroxide, which may require relocation and internal proton transfer (by a rearrangement) before it is able to be displaced by H_2 or CO. This leads to a revised proposal (Scheme 3) in which Ni_u* (Ni−A) is identified as O, Ni_u−S (Ni−SU) is identified as R, and a new species P is invoked. Interestingly, the results of Carepo et al.[21] (who found that ^{17}O hyperfine in NiA originated from labeled solvent H_2O) could be explained if a water molecule is also trapped in the active site and can somehow exchange O atoms with peroxide. This of course is highly speculative.

Scheme 3. Revised General Scheme of Reactions Showing That Activation of the Unready State of [NiFe]-Hydrogenase Involves *Two* Intermediates that are Formally at the Ni(II) State and Which Lead Back to Unready Rather than Produce Ready (Ni_r* or Ni−B) When Reoxidized by Electron Removal



It therefore follows that the role of H_2 or CO is to render the preceding equilibria *irreversible*, that is, to provide the final commitment stage of reaction. The fact that CO, otherwise established as an inhibitor of hydrogenases, is actually an *activator* is perhaps significant. An early study by Le Gall and co-workers suggested[37] that CO activates [NiFe]- hydrogenase, but the experiments were carried out under much less well defined conditions than we are now able to exploit with electrochemical control.

Conclusions

We can now summarize the new knowledge we have gained from this investigation about how *Allochromatium vinosum* [NiFe]-hydrogenase is activated. First, activation of Unready (Ni_u* or Ni−A) involves three distinct steps: a rapid redox pre-equilibrium in which Ni is reduced, a slow, rate-determining (and reversible) step that interchanges the [O] species without releasing it (thus implicating multiple forms), and a final stage requiring H_2 that commits the entire sequence to produce active enzyme. Second, reduction of the Unready Ni(III) state leads to more than one [O]-containing species, any of which might escape structural or spectroscopic detection. Third, the role of H_2 in activating the Unready state of hydrogenase is (a) to render the reductively activated transformation *irreversible*, and (b) to enable activation to occur at a higher potential, although exactly how this occurs remains unresolved. Like H_2, CO also acts by rendering the reductive transformation irreversible, and this suggests that the final stage is a displacement process. There is ample evidence that exogenous CO binds to the Ni atom,[12,13] but in order to do this, any ligand that remains close to the Ni would probably be moved. In this way, we might envisage the final departure of the [O] species, perhaps, as a peroxide. If so, the peroxide should be detectable as a stoichiometric product,

(45) See, for example: Dascent, W. E. *Inorganic Energetics*, 2nd ed.; Cambridge University Press: New York, 1982.

and we are considering ways in which this measurement could be achieved given that it would be released into a reducing environment. It is important to note that our results make it extremely unlikely that a special ligand, such as a small sulfur-containing molecule, is released into the cell as this would never return to the active site on the following cycle.

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