J|A|C|S
COMMUNICATIONS

Published on Web 05/18/2006

Rapid and Reversible Reactions of [NiFe]-Hydrogenases with Sulfide

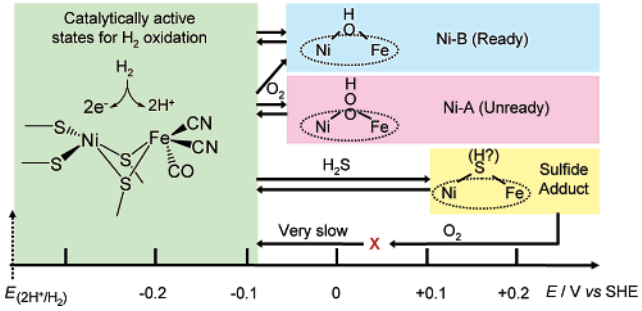
Kylie A. Vincent,† Natalie A. Belsey,† Wolfgang Lubitz,‡ and Fraser A. Armstrong*,†

Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR, U.K., and Max-Planck-Institut für Bioanorganische Chemie, P.O. Box 101365, D-45413 Mülheim an der Ruhr, Germany

Received March 13, 2006; E-mail: fraser.armstrong@chem.ox.ac.uk

Microbial H_2 oxidation is challenged by small molecules that block or destroy the active sites of hydrogenases, with examples including O_2 and CO, and also H_2S produced by sulfate-reducing bacteria.[1] Such competition has implications for interpreting structures and spectroscopic data since although X-ray crystal structures have been solved for several *Desulfovibrio* [NiFe]-hydrogenases,[2−9] puzzling differences have been noted, especially with regard to additional sulfur species in the active site of the enzyme from *D. vulgaris* Miyazaki F (MF). From crystallography of the oxidized form, Higuchi and co-workers assigned a sulfur atom in a bridging position between Fe and Ni.[6] Detection of H_2S on incubation of as-isolated enzyme with H_2 and electron donors was consistent with release of sulfide from >20% of enzyme molecules,[7,9] and X-ray crystallography showed that the bridging S atom was absent in the reduced form.[8] In contrast, crystal structures of *D. gigas* and *D. fructosovorans* enzymes revealed a bridging oxygen species in the active site[2−4] (assigned as $-OH^-$ in "Ready"/Ni−B enzyme, or probably $-OOH^-$ in "Unready"/Ni−A enzyme), and EPR/ENDOR spectra of *A. vinosum* and *D. gigas* enzymes as well as (most recently) MF itself have also supported an oxygen species in the bridging position.[10] In all cases, the bridging ligand must be removed to activate the enzyme. To illustrate these proposals, and in advance of the conclusions drawn in this communication, we refer to Scheme 1.

Scheme 1. Proposals for Potential-Dependent Inactivation and Reactivation Reactions of [NiFe]-Hydrogenases, Including Conditions for Forming the Sulfide Adduct Described Herein (approximate potential regions apply for 45 °C, pH 6, 1 bar H_2, scan rate 1 mv s^{-1}, and refer to *D. vulgaris* MF [NiFe]-hydrogenase)



We have addressed the S versus O issue using protein film voltammetry (PFV)[11] to track the species formed by sulfide addition to [NiFe]-hydrogenases during catalysis under H_2. PFV probes reactions of enzymes adsorbed on an electrode, under precise potential control, and studies on hydrogenases have defined potential profiles for catalytic activity and sensitivities to O_2.[12,13] Here we establish that [NiFe]-hydrogenases from *D. vulgaris* MF, *D. gigas*, *D. fructosovorans*, and *A. vinosum* all react reversibly with sulfide, but within a narrow potential window that easily renders the products elusive.

† University of Oxford.
‡ Max-Planck-Institut für Bioanorganische Chemie.

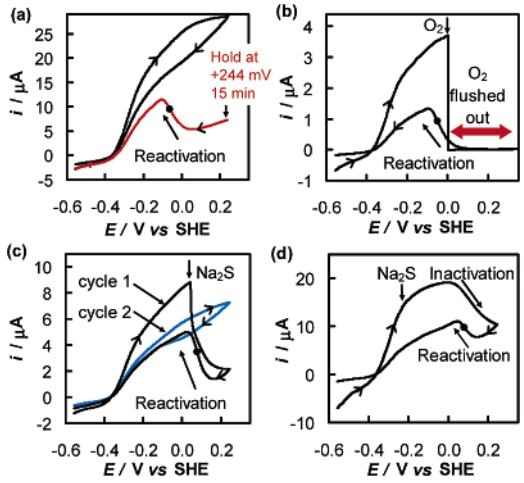


Figure 1. Voltammograms for *D. vulgaris* MF [NiFe]-hydrogenase showing the effects of inhibitors or inactivators. Other conditions: pH 6.0, 45 °C, 1 bar H_2, electrode rotation rate 2500 rpm, scan rate 3 mV s^{-1}.

The voltammograms in Figure 1 demonstrate the existence of an inactive form of *D. vulgaris* MF [NiFe]-hydrogenase formed rapidly by reaction with sulfide that is *distinct* from inactive states generated in the absence of extraneous sulfur species.

As with other [NiFe]-hydrogenases, inactive states of the *D. vulgaris* MF enzyme are formed by exposure to non-oxygenic high-potential oxidants (or high electrode potential, Figure 1a) or to O_2 (Figure 1b). Reactivation is observed electrochemically as a restoration of the electrocatalytic current (H_2 oxidation rate) as the potential is lowered. We have previously defined a "switch" potential (E_{switch}) associated with reductive activation as the inflection point of current ascent.[12] To relate to a thermodynamic potential, the scan rate must be slow compared to the reactivation rate yet fast relative to subsequent reactions. Importantly, all scan rates in Figure 1 are identical and thus allow direct comparison. At 3 mV s^{-1}, E_{switch} (●) is measured as -50 ± 10 mV after anaerobic (a) or aerobic (b) inactivation. In Figure 1c, Na_2S (recrystallized anaerobically and freshly prepared as a stock, pH 6) was injected (final concentration 1 mM) at +40 mV on the forward sweep, a potential at which the enzyme is predominantly active. The sharp drop in activity indicates rapid inhibition by sulfide. Importantly, reactivation on the return sweep occurs with $E_{switch} = +80$ mV, that is, about 130 mV more positive than that in Figure 1a and b, confirming that sulfide addition generates a new state. By the second cycle, the sulfide concentration has dropped substantially as H_2S (p*K* 6.9) is flushed away[14] and the new species is absent (note some film loss occurs). The modifying sulfide has clearly been removed from the enzyme and surrounding solution, so it cannot return. In Figure 1d, sulfide is injected at a lower potential, −200 mV, but still no inactivation occurs until the potential is increased above +40 mV. Electrode potential is thus critical in forming the sulfide adduct, and reactions would

10.1021/ja061732f CCC: $33.50

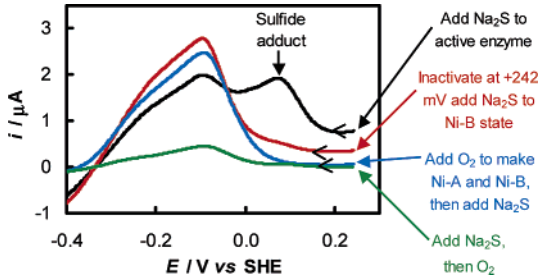


Figure 2. Voltammograms (1 mV s^{-1}) for *D. vulgaris* MF [NiFe]-hydrogenase showing recovery of activity after different pretreatments on a sweep toward negative potentials. Other conditions as for Figure 1.

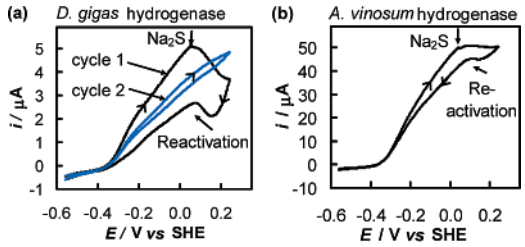


Figure 3. Cyclic voltammograms, 3 mV s^{-1}, for (a) *D. gigas* and (b) *A. vinosum* [NiFe]-hydrogenases showing the effect of sulfide on enzyme activity. Other conditions as for Figure 1.

easily be overlooked or mis-interpreted were this parameter not controlled precisely. Experiments at higher pH (up to pH 8) show much slower inactivation, and E_{switch} becomes more negative. Experiments carried out with 10% H_2 in N_2 give the same results.

Figure 2 shows recovery profiles (scans in negative direction) following addition of sulfide before or after exposure to oxidants, either anaerobic (positive electrode potential) or O_2. In all cases, the enzyme is first fully activated at −558 mV.

The black line tracks the reactions occurring after sulfide is added to active *D. vulgaris* MF [NiFe]-hydrogenase under 1 bar H_2 immediately after stepping to +242 mV. The cell headspace is flushed with H_2 for 500 s to displace H_2S from solution, then reactivation of the sulfide adduct is observed at $E_{switch} = +110$ mV. The sulfide adduct is thus stable for an oxidized form of the enzyme even when exogenous sulfide is removed. The slower scan rate in Figure 2 also allows more time for oxidative inactivation after removal of sulfide, and the second reactivation appears as expected at around −50 mV.

The red line in Figure 2 shows a sweep recorded after reaction of sulfide with anaerobically inactivated enzyme (15 min, +242 mV, giving Ni−B). Most of the sample activates at about −50 mV; the slight reactivation current at +100 mV is attributable to sulfide reacting with a tiny fraction of the sample that never entered the Ni−B state. We conclude that Ni−B does not react with sulfide; hence the site of attack is the active site rather than an Fe−S cluster.

The blue line shows the result when sulfide is introduced to enzyme that has been pretreated with O_2-saturated buffer under N_2 at +242 mV to generate a mixture of Ni−A and Ni−B.[12d] After flushing out O_2, first with N_2 then H_2, and injecting sulfide, the scan reveals only the low-potential recovery showing that the sulfide adduct is not formed from Ni−A.

The green trace shows the first sweep after the following sequence: sulfide was added immediately after a step to +242 mV; the cell was flushed with H_2 for 500 s; O_2 was injected, then removed by flushing with H_2 for 1000 s and exchanging the buffer several times. The sulfide species has virtually vanished, and there is little reactivation at more negative potential, although the second cycle (not shown) shows further recovery of activity. Thus the sulfide adduct reacts *further* with O_2 to produce a species that is kinetically distinct from the one formed when O_2 is added to active enzyme. The rate of reactivation is significantly slower than for Ni−A or Ni−B states.

Other hydrogenases react with sulfide: *D. gigas* (Figure 3a) and *D. fructosovorans* enzymes (data not shown) show similar although slower reactivity compared to that of the *D. vulgaris* MF enzyme; however *A. vinosum* hydrogenase (Figure 3b) reacts much more slowly.

Referring back to Scheme 1, we summarize the results with specific reference to *D. vulgaris* MF [NiFe]-hydrogenase. Sulfide reacts with active enzyme to give an adduct identifiable with the "μ-sulfido" species reported by Higuchi and co-workers.[6] Retardation of adduct formation at higher pH suggests that sulfide enters as H_2S rather than SH^-. The sulfide ligand is released rapidly by reduction but also reacts with O_2 to give a species (X), perhaps an S−O adduct, that activates very slowly. By analogy with oxygen species, the S^{2-}/HS^- ligand is retained only if the active site is oxidized (Ni(III)); further, because the sulfide adduct has a relatively high potential, its biological relevance is unclear. Nevertheless, isolated enzyme exposed to sulfide at appropriate redox potentials during cell disruption could exist, at least partially, in a sulfur-trapped state and could be detected crystallographically. Our direct and alternative perspective on reactions of hydrogenases with sulfide is therefore highly relevant to spectroscopic and crystallographic studies.

Acknowledgment. The authors thank C. Fichtner for preparation of *D. vulgaris* MF hydrogenase, R. Cammack for samples of *D. gigas* and *D. fructosovorans* hydrogenases, S. P. J. Albracht for assistance with purification of *A. vinosum* hydrogenase, and J. Fontecilla-Camps for useful discussions. This research was supported by the BBSRC (BB/D52222X/1).

References

(1) Heidelberg, J. F.; Seshadri, R.; Haveman, S. A.; Hemme, C. L.; Paulsen, I. T.; Kolonay, J. F.; Eisen, J. A.; Ward, N.; Methe, B.; Brinkac, L. M.; Daugherty, S. C.; Deboy, R. T.; Dodson, R. J.; Durkin, A. S.; Madupu, R.; Nelson, W. C.; Sullivan, S. A.; Fouts, D.; Haft, D. H.; Selengut, J.; Peterson, J. D.; Davidsen, T. M.; Zafar, N.; Zhou, L.; Radune, D.; Dimitrov, G.; Hance, M.; Tran, K.; Khouri, H.; Gill, J.; Utterback, T. R.; Feldblyum, T. V.; Wall, J. D.; Voordouw, G.; Fraser, C. M. *Nat. Biotechnol.* **2004**, *22*, 554.

(2) (a) Volbeda, A.; Charon, M. H.; Piras, C.; Hatchikian, E. C.; Frey, M.; Fontecilla-Camps, J. C. *Nature* **1995**, *373*, 580. (b) Volbeda, A.; Garcia, E.; Piras, C.; De Lacey, A. L.; Fernandez, V. M.; Hatchikian, E. C.; Frey, M.; Fontecilla-Camps, J. C. *J. Am. Chem. Soc.* **1996**, *118*, 12989.

(3) Volbeda, A.; Martin, L.; Cavazza, C.; Matho, M.; Faber, B. W.; Roseboom, W.; Albracht, S. P. J.; Garcin, E.; Rousset, M.; Fontecilla-Camps, J. C. *J. Biol. Inorg. Chem.* **2005**, *10*, 239.

(4) Montet, Y.; Amara, P.; Volbeda, A.; Vernede, X.; Hatchikian, E. C.; Field, M. J.; Frey, M.; Fontecilla-Camps, J. C. *Nat. Struct. Biol.* **1997**, *4*, 523.

(5) Matias, P. M.; Soares, C. M.; Saraiva, L. M.; Coelho, R.; Morais, J.; Le Gall, J.; Carrondo, M. A. *J. Biol. Inorg. Chem.* **2001**, *6*, 63.

(6) Higuchi, Y.; Yagi, T.; Yasuoka, N. *Structure* **1997**, *5*, 1671.

(7) Higuchi, M.; Yagi, T. *Biochem. Biophys. Res. Commun.* **1999**, *255*, 295.

(8) Higuchi, Y.; Ogata, H.; Miki, K.; Yasuoka, N.; Yagi, T. *Structure* **1999**, *7*, 549.

(9) Ogata, H.; Hirota, S.; Nakahara, A.; Komori, H.; Shibata, N.; Kato, T.; Kano, K.; Higuchi, Y. *Structure* **2005**, *13*, 1635.

(10) (a) van der Zwaan, J. W.; Coremans, J. M. C. C.; Bouwens, E. C. M.; Albracht, S. P. J. *Biochim. Biophys. Acta* **1990**, *1041*, 101. (b) Carepo, M.; Tierney, D. L.; Brondino, C. D.; Yang, T. C.; Pamplona, A.; Telser, J.; Moura, I.; Moura, J. J. G.; Hoffman, B. M. *J. Am. Chem.* Soc. **2002**, *124*, 281. (c) van Gastel, M.; Stein, M.; Brecht, M.; Schröder, O.; Lendzian, F.; Bittl, R.; Ogata, H.; Higuchi, Y.; Lubitz, W. *J. Biol. Inorg. Chem.* **2006**, *11*, 41.

(11) Vincent, K. A.; Armstrong, F. A. *Inorg. Chem.* **2005**, *44*, 798.

(12) (a) Jones, A. K.; Lamle, S. E.; Pershad, H. R.; Vincent, K. A.; Albracht, S. P. J.; Armstrong, F. A. *J. Am. Chem. Soc.* **2003**, *125*, 8505. (b) Vincent, K. A.; Cracknell, J. A.; Lenz, O.; Zebger, I.; Friedrich, B.; Armstrong, F. A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 16951. (c) Vincent, K. A.; Parkin, A.; Lenz, O.; Albracht, S. P. J.; Fontecilla-Camps, J. C.; Cammack, R.; Friedrich, B.; Armstrong, F. A. *J. Am. Chem. Soc.* **2005**, *127*, 18179. (d) Lamle, S. E.; Albracht, S. P. J.; Armstrong, F. A. *J. Am. Chem. Soc.* **2004**, *126*, 14899.

(13) Léger, C.; Dementin, S.; Bertrand, P.; Rousset, M.; Guigliarelli, B. *J. Am. Chem. Soc.* **2004**, *126*, 12162.

(14) Sulfide species in solution could be detected by an oxidation current above 0.4 V, which becomes very small after flushing with H_2 for 5 min.

JA061732F