Communications to the Editor

A Reversible Photoconversion between the Carbon Monoxide Induced Axial 2.06 and the Rhombic 2.10 EPR Signals of the Periplasmic Hydrogenase from *Desulfovibrio vulgaris*[1]

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This communication reports the evidence for a reversible photodissociation of carbon monoxide from a non-heme iron cluster of the *Desulfovibrio* (*D.*) *vulgaris* [Fe] hydrogenase. Both the unligated and the CO-bound states of the enzyme exhibit unique electron paramagnetic resonance (EPR) spectra, and the photodissociation process can be followed by the EPR technique.

Hydrogenases are a group of enzymes which catalyze the oxidation of molecular hydrogen and/or the reduction of proton. According to their metal contents, hydrogenases may be categorized into *the nickel-containing hydrogenases* and *the* [*Fe*] *hydrogenases*, which contain only the iron atoms.[2,3] The periplasmic hydrogenase of *D. vulgaris* (Hildenborough NCIB 8303) is composed of two subunits with molecular masses of about 45.8 and 10.5 kDa and contains two ferredoxin-type [4Fe-4S] clusters and an iron-sulfur cluster of undefined structure.[4-7] The function of the two ferredoxin-type clusters is thought to be electron transfer while the third cluster is believed to be the hydrogen-binding site. *D. vulgaris* hydrogenase is highly sensitive to inhibition by CO.[8,9] Previous investigations[6,10] have shown that when the reduced hydrogenase was exposed to CO an axial EPR signal with resonances at $g_{\parallel} = 2.06$ and $g_{\perp} = 2.01$ (the axial 2.06 signal) is induced. Stoichiometric amounts of this axial 2.06 signal (approximately one spin per molecule) could be generated with a low concentration of CO.[10] This axial 2.06 signal was reported to be photosensitive,[10] and we now have observed that at temperatures below 10 K this signal can be reversibly converted by light into a rhombic signal with resonances at g = 2.10, 2.04, and 2.00 (the rhombic 2.10 signal), found in the active hydrogenase. It should be noted that the photosensitivity of the axial 2.06 signal reported earlier[10] was detected at temperatures above 20 K, where a different photoinduced process was observed.

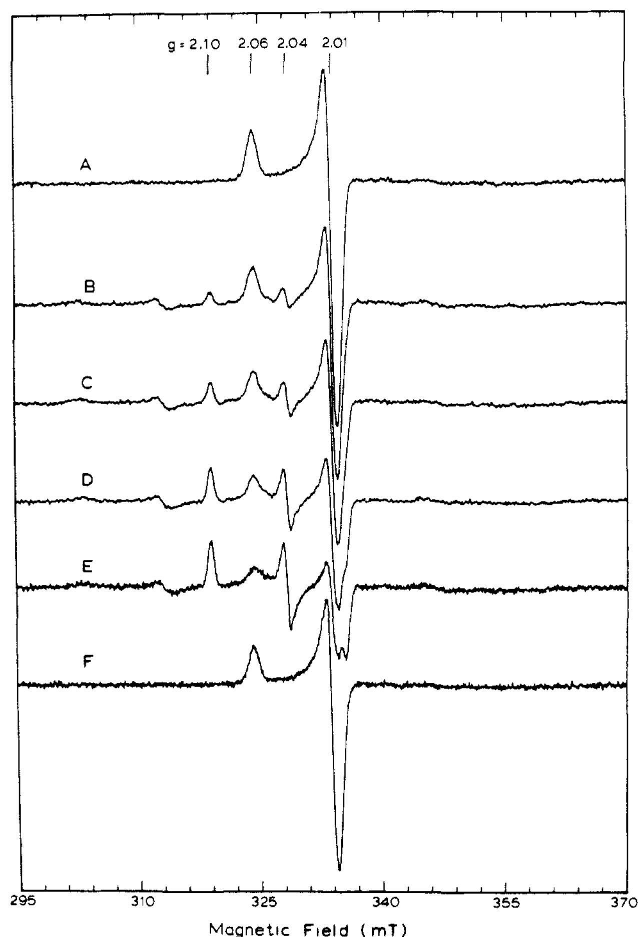

Figure 1. EPR spectra of *D. vulgaris* hydrogenase showing the effect of photoirradiation. Experimental conditions: temperature, 9 K; microwave frequency, 9.44 GHz; microwave power, 0.2 μW; modulation amplitude, 1.0 mT; receiver gain, 5×10^5. Spectra: (A) hydrogen-reduced enzyme treated with CO; (B) 20 min, (C) 40 min, (D) 80 min, and (E) 160 min irradiation with light; (F) warming the irradiated sample up to 150 K for 10 min and cooling back down to 9 K.

The *D. vulgaris* hydrogenase was purified as previously described.[6] The specific activity of the purified enzyme in the hydrogen evolution assay was 3800 ± 250 μmol of H_2/min/mg of protein. The EPR sample (140 μM protein in 50 mM Tris-HCl buffer, pH = 7.6) was reduced under a hydrogen atmosphere for about 30 min. A 10% volume of CO with respect to the total volume of the sealed EPR tube was injected into the reduced sample and incubated for 30 min before freezing with liquid nitrogen. An EPR spectrum of the CO-reacted hydrogenase (Figure 1A) was then recorded at 9 K on a Bruker ER 200D-SRC spectrometer equipped with an Oxford Instruments ESR 910 continuous flow cryostat. The axial 2.06 signal with a spin concentration of approximately 0.8 spin per molecule was observed. The sample, inside the EPR cavity at 9 K, was then subjected to the irradiation of a focused light beam from a 200-W mercury arc lamp (Oriel Optics Corp, C-60-30). After each selected interval of irradiation an EPR spectrum was taken. The results are shown in Figure 1 (traces B (20 min), C (40 min), D (80 min), and E (160 min)). These spectra indicated that irradiation of the CO-reacted sample at low temperature caused a decrease of intensity of the axial 2.06 signal and simultaneous appearance of the rhombic 2.10 signal.[11] After 3 h of irradiation time, the

(1) Supported in part by grants from the National Institutes of Health (GM 32187, GM 34903, and DK 01135) and under contract no. DEAS-09-80 ER 10499-A002.

(2) Adams, M. W. W.; Mortenson, L. E.; Chen, J. S. *Biochim. Biophys. Acta* **1981**, *594*, 105-176.
(3) LeGall, J.; Moura, J. J. G.; Peck, H. D., Jr.; Xavier, A. V. Iron-Sulfur Proteins. Spiro, T. G., Ed.; J. Wiley & Sons: New York, 1982; Vol. 4, pp 177-248.
(4) Voordouw, G.; Brenner, S. *Eur. J. Biochem.* **1985**, *148*, 515-520.
(5) Hagen, W. R.; van Berkel-Arts, A.; Krüse-Wolters, K. M.; Voordouw, G.; Veeger, C. *FEBS Lett.* **1986**, *203*, 59-63.
(6) Huynh, B. H.; Czechowski, M. H.; Krüger, H. J.; DerVartanian, D. V.; Peck, H. D., Jr.; LeGall, J. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3728-3732.
(7) Grande, H. J.; Dunham, W. R.; Averill, B.; Van Dijk, C.; Sands, R. H. *Eur. J. Biochem.* **1983**, *136*, 201-207.
(8) Krasna, A. I.; Rittenberg, D. *Proc. Natl. Acad. Sci. U.S.A.* **1954**, *40*, 225-227.
(9) Yagi, T.; Honya, M.; Tamiya, N. *Biochim. Biophys. Acta* **1968**, *153*, 699-705.
(10) Patil, D. S.; Czechowski, M. H.; Huynh, B. H.; LeGall, J.; Peck, H. D., Jr.; DerVartanian, D. V. *Biochem. Biophys. Res. Commun.* **1986**, *137*, 1086-1093.

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rhombic 2.10 signal reached a maximum intensity corresponding to ~0.5 spin/molecule, while the axial 2.06 signal was reduced to a minimum value of 0.3 spin/molecule. Since the light beam was focused onto only a part of the sample, complete conversion of the axial 2.06 signal to the rhombic 2.10 signal was not expected. Most interestingly, this photoinduced process was found to be reversible by warming up the sample and letting it stand for 10 min at 150 K. An EPR spectrum recorded at 9 K after warming is shown in Figure 1F. The rhombic 2.10 signal disappeared, and the axial 2.06 signal recovered approximately 80% of its original intensity. Storing the sample in liquid nitrogen for 24 h restored the full intensity of the axial 2.06 signal. For a given sample the irradiation-warming cycle could be repeated several times with consistently reproducible results.

The rhombic 2.10 signal could also be induced by partially oxidizing the reduced hydrogenase under an argon atmosphere.[12] In order to show that CO is involved in the above mentioned photoconversion process, we produced a partially reoxidized hydrogenase sample under argon exhibiting the 2.10 signal and subjected it to the higher temperature (150 K). It was found that the argon-induced rhombic 2.10 signal retained its full intensity and did not convert into the axial 2.06 signal.

Both the axial 2.06 and the rhombic 2.10 signals have been observed previously in the [Fe] hydrogenases isolated from *D. vulgaris*,[6,12] *Clostridium(C.) pasteurianum*,[13,14] and *Megasphaera elsdenii*.[15] In the case of *C. pasteurianum* hydrogenase I the axial 2.06 signal was induced by reacting CO with preparations which exhibited the rhombic 2.10 signal.[13] The axial 2.06 signal was shown to represent a CO-bound cluster by the observation of ^{13}C resonances in an ENDOR study of the ^{13}CO-treated enzyme.[16] In the case of the [Fe] hydrogenase from *D. vulgaris*, the relationship between these two signals has not been extensively studied. It should be noted in this regard that the axial 2.06 signal can also be induced by chemical oxidants.[6] Furthermore, the physiological significance of the axial 2.06 signal has long been a controversy. It has been proposed that the axial 2.06 signal observed in *D. vulgaris* hydrogenase was caused by the unintentional exposure of the enzyme to oxygen and represented irreversibly inactivated hydrogenase.[12] However, our recent investigation has shown that *D. vulgaris* hydrogenase with the fully developed axial 2.06 signal (induced by CO) could be reversibly activated.[17]

The present study suggests that the CO-induced 2.06 and the rhombic 2.10 signals observed in *D. vulgaris* hydrogenase originate from the same iron-sulfur cluster with the axial 2.06 signal representing the putative CO-bound cluster and the rhombic 2.10 signal, the unligated cluster. The effect of irradiation is to flash off the bound CO. The facts that the rhombic 2.10 signal retains its intensity at low temperature and that it can be converted back to the axial 2.06 signal by raising the temperature suggest that the flashed-off CO remains in the protein matrix but is separated from the cluster by an energy barrier(s). At higher temperatures, the CO is capable of crossing the barrier and recombining with the cluster, a plausible mechanism which bears similarity with that described for the CO binding of myoglobin where certain energy barriers were postulated.[18] Since CO is a competitive inhibitor of *D. vulgaris* hydrogenase, it is apparent that both the axial 2.06 and the rhombic 2.10 signals are of physiological significance. In general, metal centers in proteins can be grouped into catalytic substrate-binding sites as well as electron-transfer centers. The observed photoreaction of the 2.06 signal demonstrates that in the [Fe] hydrogenase from *D. vulgaris* a specific iron-sulfur cluster is involved in ligand binding. The fact that the 2.06 and the 2.10 signals are commonly observed in the [Fe] hydrogenases suggests further that the [Fe] hydrogenases must share this unique active center.

(11) In addition to the rhombic 2.10 signal, a weak EPR signal with g values at 2.21 and 2.15 was also detected upon irradiation. However, its intensity did not increase with irradiation time. A control experiment on buffer solution indicated that this weak signal was intrinsic to the protein sample.

(12) Hagen, W. R.; van Berkel-Arts, A.; Krüse-Wolters, K. M.; Dunham, W. R.; Veeger, C. *FEBS Lett.* **1986**, *201*, 158-162.
(13) Erbes, D. L.; Burris, R. H.; Orme-Johnson, W. H. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 4795-4799.
(14) Chen, J. S.; Mortenson, L. E.; Palmer, G. Iron and Copper Proteins. Yasunobu, K. T., Mower, H. F., Hayaishi, O., Eds.; Plenum Press: New York, 1976; pp 68-82.
(15) Van Dijk, C.; Grande, H. J.; Mayhew, S. G.; Veeger, C. *Eur. J. Biochem.* **1980**, *107*, 251-261.
(16) Telser, J.; Benecky, M. J.; Adams, M. W. W.; Mortenson, L. E.; Hoffman, B. M. *J. Biol. Chem.* **1986**, *260*, 13536-13541.
(17) Patil, D. S.; He, S. H.; DerVartanian, D. V.; LeGall, J.; Huynh, B. H.; Peck, H. D., Jr. *FEBS Lett.* **1988**, *228*, 85-88.
(18) Austin, R. H.; Beeson, K. W.; Eisenstein, L.; Frauenfelder, H. *Biochemistry* **1975**, *14*, 5355-5373.

A General Treatment of Hydrogen Bond Complexation Constants in Tetrachloromethane

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We wish to report a generalized treatment applicable to 1:1 hydrogen bond complexation constants (as log K values) for a large number of acid:base pairs in solvent tetrachloromethane. Recently, we have shown[1] that when log K values for a series of acids (hydrogen bond donors) against a given reference base are plotted versus log K values for the acid series against any other reference base, there results a set of lines that intersect at a point where log $K = -1.1$, when equilibrium constants are expressed in molar concentration units. Because the order of solute hydrogen bond acidity is independent of the reference base (for exceptions see

(1) Abraham, M. H.; Duce, P. P.; Grellier, P. L.; Prior, D. V.; Morris, J. J.; Taylor, P. J. *Tetrahedron Lett.* **1988**, *29*, 1587.

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