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The relationship between activity and the axial $g = 2.06$ EPR signal induced by CO in the periplasmic (Fe) hydrogenase from *Desulfovibrio vulgaris*

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The effect of exposure to carbon monoxide on the activity of the (Fe) hydrogenase from *Desulfovibrio vulgaris* has been determined. Concentrations of carbon monoxide which completely inhibit hydrogenase activity and induce formation of the axial $g = 2.06$ EPR signal up to 0.8 spin/molecule do not cause irreversible inhibition of the (Fe) hydrogenase.

Hydrogenase; Carbon monoxide; ESR; (*Desulfovibrio vulgaris*)

1. INTRODUCTION

Aerobically purified periplasmic hydrogenase [(Fe) hydrogenase] from the sulfate-reducing anaerobe, *Desulfovibrio vulgaris,* has a calculated molecular mass of approx. 56 kDa and is composed of two subunits (46 kDa, 10.5 kDa) [1,2]. Spectroscopic and cluster-extrusion data indicate the prosthetic groups to be two ferredoxin-type [4Fe-4S] clusters and an iron cluster with abnormal spectroscopic properties [3,4]; however, the total iron content has been reported to vary between 11 and 16 atoms per molecule [3,5]. The unique sensitivity of the hydrogenase activity found in *D. vulgaris* to CO was first recognized by Krasna and Rittenberg [6] but its significance was not appreciated until the presence of multiple hydrogenases with different biochemical properties in this bacterium was reported [7]. The (Fe) hydrogenase has now been shown to be highly sensitive to inhibition by CO in contrast to the nickel-containing hydrogenases [8] and we have reported that an axial EPR signal ($g_{\parallel} = 2.06$, $g_{\perp} = 2.01$) is induced to approx. 1 spin/molecule by low concentrations of CO [9]. The signal was detected at 77 K and was readily reversed to a reduced ferredoxin-type $g = 1.94$ signal, either by exchanging the CO with H_2 or by illuminating the frozen (Fe) hydrogenase in the EPR cavity. An identical or similar axial $g = 2.06$ signal is induced by the presence of oxidizing agents [tetraheme cytochrome c_3 or dichlorophenol indophenol (DCPIP)] but the exact relationship between the two $g = 2.06$ EPR signals has not been resolved. Here, we report further studies concerning the influence of CO on the reduced (Fe) hydrogenase and the specific activity measurements accompanying the EPR changes, which clearly indicate that CO at low concentrations does not irreversibly inactivate the (Fe) hydrogenase.

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2. MATERIALS AND METHODS

D. vulgaris (Hildenborough, NCIB 8303) was grown on a lactate-sulfate medium and purified as in [3]. The enzyme purity was checked by SDS-PAGE on a 12.5% slab gel which show-

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ed the presence of two subunits (approx. 45 kDa, 11 kDa). No traces of impurities were detected.

The EPR spectra were recorded on a Bruker ER 200D-SRC spectrometer. Spin quantitations were performed using myoglobin azide (220 μM) as a standard and applying the procedures described by Aasa and Vanngord [10]. The hydrogenase samples (120 μM, pH 7.6, 200 mM Tris-HCl buffer) were prepared anaerobically by employing a specially designed glass manifold equipped with a vacuum pump and H_2/Ar gas source. Gases were rendered oxygen-free by passing over a heated copper catalyst and/or a 10% solution of sodium dithionite. The manifold was evacuated and then purged with the desired gas. Enzyme samples in EPR tubes stoppered with serum-seals were attached to the manifold through a syringe needle. After 2–3 evacuation-purge cycles, a fine stream of H_2 gas was allowed to pass over the enzyme solution for about 30 min. During this period, occasional stirring by gentle tapping of the EPR tube was adequate to effect complete reduction of the (Fe) hydrogenase. For comparison, an alternative procedure [11] was adapted by inserting a tiny stir-bar into the EPR tube and agitating by means of a magnetic stirrer. Although the precession of the stir-bar effected a rapid mixing of the enzyme there was no improvement in the rate of reduction by hydrogen. Three separate samples were reduced simultaneously and an aliquot from each was tested for activity after reduction. One of the tubes was frozen for EPR and to each of the remaining two reduced samples, CO was injected to give the desired concentration (% v/v; percent volume of CO injected with respect to the total volume of the sealed EPR tube) [9]. The tubes were allowed to stand for 30 min with occasional shaking and an aliquot was withdrawn for determination of specific activities and the samples were then frozen and EPR spectra recorded. Another sample of enzyme was treated with a fine stream of CO gas, purged for 30 min to ensure an excess dose at one atm and frozen for EPR after determining specific activity.

Specific activity was measured by a modification of the method of Peck and Gest [12]. For the assay, EPR samples were diluted 1000-fold with argon-bubbled phosphate buffer (10 mM). Since a concentrated enzyme was used for EPR an adjustment of concentration was necessary for the assay of hydrogenase activity. The diluted enzyme (5 μl containing 50 ng protein) was assayed immediately by injecting it into a serum-stoppered vial containing the reaction mixture, which was composed of potassium phosphate buffer (pH 7.6, 0.1 M), excess sodium dithionite (15 mM), reduced methyl viologen (1 mM) and bovine serum albumin (1 mg) in a final volume of 3 ml. The H_2 evolved in the reaction was determined by injecting 250 μl of the gas phase into a Varian 4600 gas chromatograph equipped with a molecular sieve 5A column maintained at 105°C (6 foot, 1/8 inch, 45/60 μm). For each enzyme sample, two sets of separate measurements were performed on duplicate vials. After initiating the reaction, gas phase injections were made at 5 min intervals over a period of 20 min. Thus, a set of eight readings were recorded for each sample. A calibration curve was constructed by injecting a known volume of pure H_2 gas into the gas chromatograph. The hydrogen production assay was monitored for the native, hydrogen-reduced, and CO-treated enzyme samples and values corresponding to the maximum rate of H_2 evolution were used to calculate the specific activities. Protein was determined by the method of Lowry et al. [13].

3. RESULTS AND DISCUSSION

In fig.1, the EPR spectra of native (as isolated) hydrogenase (a), hydrogen-reduced hydrogenase (b) and CO-treated hydrogen-reduced hydrogenase, 10% CO (c) and 100% CO (d) are shown. The native (Fe) hydrogenase shows a weak $g = 2.02$ isotropic EPR signal which integrates to approx. 0.02 spin/molecule and has been reported previously [3,4]. This signal is characteristic of a three-iron center but, because of its low spin quantitation, does not appear to be of catalytic signifi-significance. There is no effect of CO on this EPR signal ($g = 1.94$) which is characteristic of two interacting (Fe_4S_4) centers and Mössbauer data support this conclusion [3,14]. Variable amounts (0.03 spin/molecule) of an axial $g = 2.06$, 2.01 EPR signal may be distinguished in the 6.5 K spectrum of the hydrogen-reduced hydrogenase [3,5] and, above 20 K, the $g = 2.06$ signal becomes the only EPR feature observed [3]. The replacement of H_2 in the hydrogen reduced hydrogenase sample by argon results in an apparent partial reoxidation of the (Fe) hydrogenase with the appearance of a rhombic signal with g values at 2.11, 2.05, and 2.00 [5]. In the presence of CO, the rhombic signal disappears with the appearance of the axial $g = 2.06$ signal (unpublished). A similar rhombic signal has been observed in partially reoxidized samples of the bidirectional [15] and uptake [16] hydrogenases from *Clostridium pasteurianum* and the bidirectional hydrogenase of *Megasphaera elsdenii* [17].

The replacement of H_2 with CO in the hydrogen-reduced hydrogenase sample results in the disappearance of the '$g = 1.94$' and a 10-fold (to 0.2 spin/molecule) enhancement of the $g = 2.06$ EPR signal (fig.1d). Similarly, injection of a lesser amount of CO (10%, v/v) into the hydrogen-reduced hydrogenase sample results in the disappearance of the $g = 1.94$ signal and the appearance of the axial $g = 2.06$ signal (fig.1c); however, the spin quantitation is now close to unity. The formation of the $g = 2.06$ signal is reversible either by replacement of the CO with H_2 or by illumination of the sample by white light [9], an observation which is reminiscent of the behavior of the nickel-containing hydrogenase from *Chromatium* [18]. In the presence of oxidizing agents such as the tetraheme cytochrome c_3 and DCPIP [3,19] the

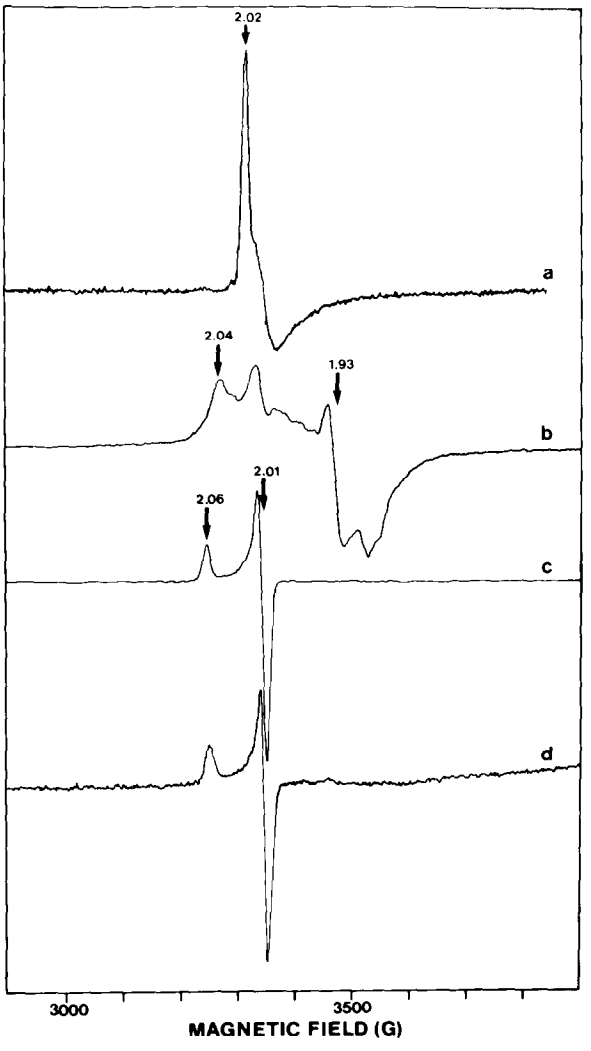

Fig.1. EPR spectra of hydrogenase from *D. vulgaris*. The sample was 120 μM in protein in 200 mM Tris-HCl buffer (pH 7.6). Spectra: (a) hydrogenase as purified; (b) sample reduced with H_2 for 30 min; (c) hydrogen-reduced sample treated with 10% (v/v) CO; (d) hydrogen-reduced sample with 100% (v/v) CO. EPR conditions: temperature, 6.5 K; microwave frequency, 9.44 GHz; microwave power, 6.3 μW for a,b, 0.63 μW for c,d; modulation amplitude, 1.0 mT; receiver gain, 8×10^5 for a,d, 3.2×10^5 for b, and 2.5×10^5 for c.

$g=1.94$ EPR signal exhibited by the hydrogen-reduced (Fe) hydrogenase disappears and is replaced by an axial $g=2.06$ signal identical to that observed in the presence of CO. It has been proposed that this conversion requires the presence of O_2 and results in the oxidative inactivation of the hydrogenase [5,20]. We have now demonstrated (table 1) that exposure of the H_2-reduced hydrogenase to CO and the formation of the $g=2.06$ EPR signal do not result in any irreversible inactivation of the hydrogenase. Even after exposure of the reduced (Fe) hydrogenase to 100% (1 atm) CO where it appears that two types of non-heme clusters may bind CO [3], there is no loss of hydrogenase activity. It should also be noted that an activation of the (Fe) hydrogenase by CO which might account for the observed results has been observed only with nickel-containing hydrogenases [21]. Based on these results, we propose that the $g=2.06$ signal induced by CO represents the reversible liganding of CO to a non-heme iron center essential for the catalytic activity of the hydrogenase, perhaps the binding site for hydrogen.

The relationship between the $g=2.06$ signals induced by CO and oxidizing agents is not immediately obvious. Studies with ^{57}Fe-enriched hydrogenase demonstrated that both signals originate from non-heme iron clusters [3] and their identity is strongly supported by the temperature dependence and hyperfine broadening of their EPR spectra. On the other hand, the induction of the EPR signal by oxidizing agents has been proposed to reflect the formation of a sulfur radical in close proximity to non-heme iron [19]. An alter-

Table 1

Recovery of (Fe) hydrogenase activity after exposure to carbon monoxide

Enzyme sample	Spec. act. (μM H_2/mg per min)		Spin concentration of the axial $g=2.06$ signal (spin/molecule) in the presence of CO
	Before CO treatment	After CO treatment	
Native	3600 ± 250	–	–
H_2-reduced	3300 ± 400	–	–
H_2-reduced + CO (10%, v/v)	3300 ± 400	3500 ± 400	0.8 ± 0.1
H_2-reduced + CO (100%, v/v, 1 atm)	3300 ± 400	3800 ± 200	0.2 ± 0.05

For conditions, see section 2. For measurement of the EPR spectrum, the hydrogenase concentration was 120 μM. For determination of hydrogenase activity, 50 ng of hydrogenase was employed for each assay

native possibility might be that CO acts as an oxidizing agent via contaminating CO dehydrogenase in the hydrogenase and the reduction of CO dehydrogenase by hydrogenase has been reported [22]; however, we have been unable to detect CO dehydrogenase activity in our hydrogenase preparations. A second major problem involves the nature of the interaction of CO with the (Fe) hydrogenase. Comparative studies of the induction of the $g=2.06$ signal by ^{12}CO and ^{13}CO did not reveal significant broadening of the $g=2.06$ signal [3]; however, ENDOR studies with the related hydrogenase I from *C. pasteurianum* demonstrate that the induction of the $g=2.06$ EPR signal is due to the covalent bonding of CO to a non-heme iron center [23]. If the same mechanism is involved in the generation of the $g=2.06$ signal in the (Fe) hydrogenase of *D. vulgaris,* then it is difficult to understand the generation of the $g=2.06$ signal by oxidizing agents in the absence of exogenous CO. The third major difference in the induction of the $g=2.06$ EPR signals involves enzymatic activity. As shown here, the inhibition of activity by CO is completely reversible (10–100% CO); however, the generation of the $g=2.06$ signal by oxidizing agents has been proposed to be due to incomplete anaerobicity and results in the irreversible inactivation of the hydrogenase [20].

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