Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 9378–9380, December 1988
Biochemistry

Hydrogen production and deuterium-proton exchange reactions catalyzed by *Desulfovibrio* nickel(II)-substituted rubredoxins

(hydrogenase)

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Communicated by Martin D. Kamen, August 2, 1988

ABSTRACT **The nickel tetrahedral sulfur-coordinated core formed upon metal replacement of the native iron in *Desulfovibrio* sp. rubredoxins is shown to mimic the reactivity pattern of nickel-containing hydrogenases with respect to hydrogen production, deuterium-proton exchange, and inhibition by carbon monoxide.**

The biological role of nickel is now well established in several and diversified biological systems (1) with relevance to hydrogen metabolism. Nickel-containing hydrogenases have been shown to possess a distinctive coordination environment of the metal center. Extended x-ray absorption fine structure (EXAFS) measurements suggested a predominant sulfur coordination around the monomeric nickel (2). However, so few monomeric thiolate compounds are currently available that they cannot be used for modeling the active site of bacterial hydrogenases (3).

Rubredoxin is the simplest protein of the iron-sulfur class; it contains one iron atom bound in a tetrahedral coordination by the sulfur atoms of four cysteinyl residues. The simple constitution of this active center as well as the low molecular mass makes this protein suitable for the synthesis of metal-substituted derivatives.

We have successfully substituted the native iron atom of rubredoxins from *Desulfovibrio* with cobalt and nickel (4). The just formed nickel and cobalt cores were then extensively characterized by a variety of spectroscopic probes including UV/visible, electron paramagnetic resonance, ^1H nuclear magnetic resonance, and magnetic circular dichroism (4, 5). These techniques indicated that a tetrahedral sulfur coordination was maintained in both Co- and Ni-substituted rubredoxins.

In this article we show that the nickel-substituted rubredoxins from three species of sulfate-reducing bacteria of the genus *Desulfovibrio* are active in both the deuterium-proton exchange reaction and in H_2 production (using dithionite-reduced methyl viologen as electron donor). The nickel-substituted rubredoxins, providing a sulfur environment for the metal center, can mimic in some aspects the bacterial hydrogenase activity.

MATERIAL AND METHODS

Preparation of Nickel-Substituted Rubredoxins. The growth conditions on a lactate/sulfate medium for *Desulfovibrio gigas* (NCIB 9332) and *Desulfovibrio desulfuricans* strains Berre-eau (NCIB 8387) and ATCC 27774 as well as the purification of their rubredoxins have been reported (6–9).

The experimental procedure used for the preparation of the aporubredoxin and the reconstitution of the active center with nickel is described elsewhere (4, 5). The apoprotein was prepared by precipitation with trichloroacetic acid and reconstituted by adding a stoichiometric amount of nickel(II) nitrate. The nickel-substituted protein was then purified by gel filtration. Metal analyses were performed by plasma emission spectroscopy with a Jarrell–Ash model 750 Atomcomp. Protein concentrations were determined by the Lowry method with bovine serum albumin as a standard (10) or by using the previously determined extinction coefficient (ε_{448nm} = 3200 $M^{-1}\cdot cm^{-1}$) (4, 5).

Deuterium-Proton Exchange and H_2 Evolution Measurements. A mass spectrometric technique using a direct membrane-inlet system has been devised for on-line analysis of dissolved gases (11, 12). The experiments were done in a reaction vessel connected via a Teflon-membrane inlet to the ion source of a VG MM model 8–80 mass spectrometer monitored by an Apple II data acquisition system (12). Activation of molecular hydrogen was determined by following either the rate of deuterium-proton exchange after saturation with the oxygen-free mixture $N_2/^2H_2$, 80:20 (monitoring mass peaks 2, 3, and 4) or the rate of hydrogen production from dithionite-reduced methyl viologen (13) (monitoring mass peak 2). All the activity determinations were made in 0.1 M potassium phosphate buffer, pH 6.8 at 32°C.

To study carbon monoxide inhibition of these activities, discrete amounts (that increased with each solution) of CO-saturated solution were successively added to the enzyme preparation in the vessel, and the activities before and after each addition were determined.

RESULTS AND DISCUSSION

Native (iron) rubredoxin, aporubredoxin, and nickel(II) chloride were used as blanks. No net hydrogen activation and deuterium-proton ($^2H_2$-H^+) exchange activity could be observed above instrumental consumption levels in any of these cases. In contrast, when Ni-substituted rubredoxins were added to the reaction vessel, a net H_2 production (Table 1) and $^2H_2$-H^+ exchange in the presence of 20% deuterium were recorded (see Figs. 1 and 2). The respective values for H_2 + H(^{2}H) evolution and for the H_2 production are indicated in Table 1 for Ni-substituted rubredoxin proteins from *D. gigas* and *D. desulfuricans* strains Berre-eau and ATCC 27774 and compared with *Desulfovibrio* hydrogenases. {A rubredoxin-like protein, desulforedoxin, was also purified from *D. gigas*. This protein was characterized by electron paramagnetic resonance and Mössbauer spectroscopies and

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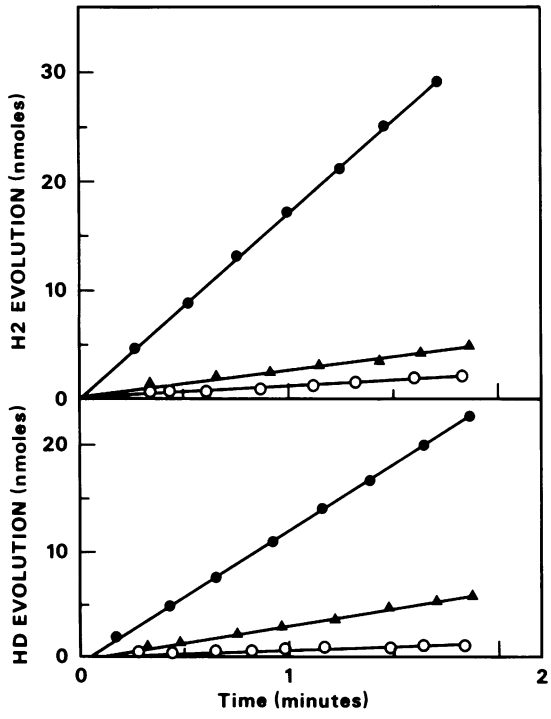

FIG. 1. H_2 and H(^{2}H) evolution in the presence of 20% $^2H_2$ by Ni-substituted rubredoxins of three organisms: *D. desulfuricans* ATCC 27774 (●), *D. desulfuricans* Berre-eau (▲), and *D. gigas* (○).

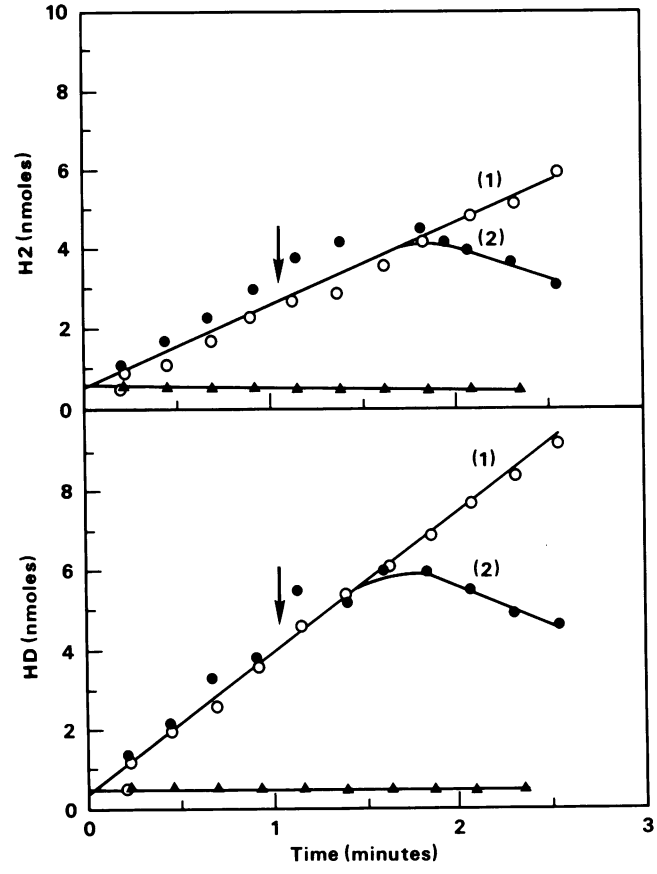

FIG. 2. H_2 and H(^{2}H) evolution by different forms of rubredoxin from *D. gigas*. ▲, Native rubredoxin; Ni-substituted rubredoxin: experiment 1, evolution without CO (○); experiment 2, 10 μM CO was added (●) at the time indicated by arrow.

shown to have a distorted FeS_4 core (9). Replacement by nickel of the native iron core yields a substituted protein with a different geometry from that of *D. gigas* Ni-rubredoxin as judged by visible and near-IR measurements (4). The protein exhibits deuterium-protein exchange rate [≈25–30 nmol of H_2 + H(^{2}H) evolved/min per mg] but is a very poor H_2-evolving system (≈1.3 nmol of H_2 produced/min per mg). The exchange process is also inhibited by CO at the same level as that of Ni-rubredoxins.}

The values obtained with the Ni-substituted rubredoxins are low when compared with the corresponding activities of bacterial hydrogenases isolated from the same sulfate-reducing species, which were 440 and 152 μmol/min per mg in H_2 production and 267 and 66 in the $^2H_2$-H^+ exchange, respectively, for the periplasmic hydrogenase from *D. gigas* and the partially purified soluble hydrogenase from *D. desulfuricans* ATCC 27774 (ref. 14 and our unpublished results).

Deuterium-proton isotope-exchange measurements have been a useful experimental tool for probing the mechanistic involvement of the catalytic redox centers (11). The initial ratio of H_2 to H_2 + H(^{2}H) produced in the exchange reaction indicates the type of cleavage undergone by the deuterium molecule (11, 14). For instance, this ratio is higher than 0.95 with PtO_2, which cleaves homolytically the $^2H_2$ molecule, whereas it is ≈0.3 for $RuCl_3$, which is known to cleave this molecule heterolytically (11–14).

H_2 to H_2 + H(^{2}H) ratios of 0.2–0.3 and 0.5–0.6, respectively, were found with the NiFe-hydrogenases [from *D. gigas* and *D. desulfuricans* ATCC 27774 (11)] and with the NiFeSe-hydrogenases (from *Desulfovibrio baculatus* DSM 1743 and *Desulfovibrio salexigens*, British Guiana) (14, 15). These differences have been discussed in terms of modulation of the exchange process by the ligands around the hydrogenase active center (15). Modified Ni-rubredoxins have ratio values of 0.45–0.60—i.e., closer to those of NiFeSe- than to those of NiFe-enzymes. These figures rather favor a heterolytic cleavage mechanism that involves formation of a hydride.

CO is a well-known inhibitor for hydrogenase activities (16). A 50% inhibition of the deuterium-proton exchange rate was obtained for the Ni-substituted rubredoxins from *D. gigas* and *D. desulfuricans* ATCC 27774 with only about 0.5 μM of CO (Fig. 3). The Ni-substituted rubredoxins are thus

Table 1. H_2 production and deuterium-proton exchange reaction in Ni-substituted rubredoxin proteins and comparison with bacterial hydrogenases

Protein	H_2 + H(^{2}H)*	H_2†	Ref.
Blank	0.6 ± 0.3	0.6 ± 0.3	This work
D. desulfuricans (ATCC 27774) Ni-Rd	29.0 ± 4.0	9.9 ± 0.9	This work
D. desulfuricans (Berre-eau) Ni-Rd	2.1	2.1	This work
D. gigas Ni-Rd	3.8 ± 0.9	7.4 ± 0.9	This work
NiFe-hydrogenase *D. gigas*	267×10^3	440×10^3	14
NiFeSe-hydrogenase *D. salexigens*	900×10^3	1830×10^3	14

All activities expressed in nmol/min per mg. Rd, rubredoxin.
*Deuterium-proton exchange.
†H_2 production.

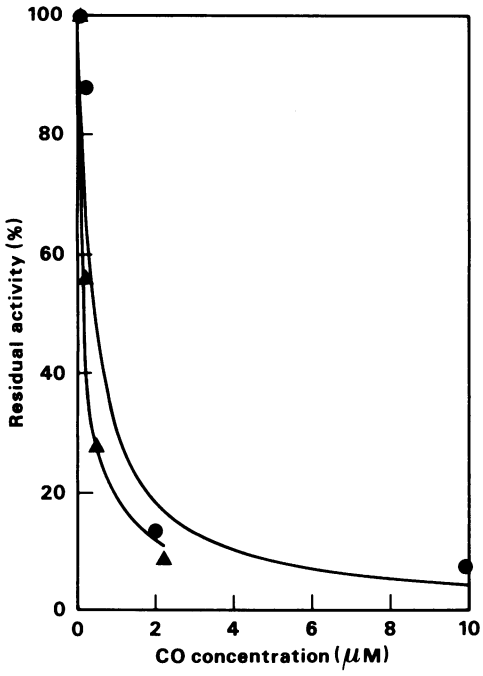

FIG. 3. CO inhibition of H_2 + H(^{2}H) evolution in the presence of 20% $^2H_2$ by Ni-substituted rubredoxin from *D. gigas* (▲) and *D. desulfuricans* ATCC 27774 (●).

more sensitive to CO inhibition than the corresponding Ni-hydrogenases, which require between 20 and 30 μM of CO for attaining 50% inhibition of the exchange reaction (17). A comparable sensitivity was found only for *Desulfovibrio vulgaris* hydrogenase, a nonnickel-containing enzyme, and for the NiSe-hydrogenase from *D. baculatus* or *D. salexigens*, which were equally inhibited with only 0.1 and 1 μM of CO, respectively (17, 18).

At present it is difficult to establish a complete mechanism for hydrogen activation by the modified rubredoxins. It is also surprising that differences are seen between the three Ni-substituted rubredoxins in terms of hydrogen activation. The most active Ni-substituted rubredoxin is that isolated from *D. desulfuricans* ATCC 27774. This rubredoxin has the peculiarity of having the shortest amino acid polypeptide chain (45 amino acids instead of the 53 of the other two) but has one more cysteine (5 cysteine residues instead of 4) (7). These differences may be significant in the respective activities observed, but further experiments are necessary to assess the implications of these differences in the mechanism of activation of the hydrogen molecule by the modified Ni-rubredoxins.

Our earlier spectroscopic studies of Ni rubredoxins by nuclear magnetic resonance (4) and magnetic circular dichroism (5) are interpretable in terms of tetragonally distorted tetrahedral thiolate coordination of Ni(II) with $S = 1$. It has been pointed out that the Ni(II) thiolate centers in hydrogenase are probably low-spin ($S = 0$), suggesting either a square planar, a square pyramidal, or an axially distorted octahedral coordination geometry (5). In spite of these differences the Ni-substituted rubredoxins, which provide a sulfur environment for the metal center and exhibit hydrogenase-like activity, are good tools for understanding the role that nickel plays in hydrogenases.

The sulfate-reducing bacteria used in this study were grown in the Association pour la Recherche en Bioenergie Solaire fermentation plant at Cadarache for which we thank R. Toci and N. Euvé. We also thank I. Pacheco and N. Galliano for their skillful technical help. This research was supported by Junta Nacional de Investigação Científica e Tecnológica, Instituto Nacional de Investigação Científica, and CEE BAP-0259-P-(TT) (J.J.G.M.) and CEE BAP-0269-F-(TT) (P.A.L.) and NSF-DMB 8602789 (J.L.).

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