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Mechanism of inhibition of NiFe hydrogenase by nitric oxide

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ABSTRACT

Hydrogenases reversibly catalyze the oxidation of molecular hydrogen and are inhibited by several small molecules including O_2, CO and NO. In the present work, we investigate the mechanism of inhibition by NO of the oxygen-sensitive NiFe hydrogenase from *Desulfovibrio fructosovorans* by coupling site-directed mutagenesis, protein film voltammetry (PFV) and EPR spectroscopy. We show that micromolar NO strongly inhibits NiFe hydrogenase and that the mechanism of inhibition is complex, with NO targeting several metallic sites in the protein. NO reacts readily at the NiFe active site according to a two-step mechanism. The first and faster step is the reversible binding of NO to the active site followed by a slower and irreversible transformation at the active site. NO also induces irreversible damage of the iron–sulfur centers chain. We give direct evidence of preferential nitrosylation of the medial [3Fe–4S] to form dinitrosyl–iron complexes.

1. Introduction

Hydrogenases are enzymes that reversibly catalyze the oxidation of molecular hydrogen into protons and electrons. As their catalytic efficiency is comparable to that of platinum [1], these enzymes have been extensively studied for their potential applications in biotechnological devices. Hydrogenases are classified on the basis of their catalytic site metal content, namely Fe, FeFe or NiFe. Standard NiFe hydrogenases, including the periplasmic heterodimeric NiFe hydrogenase from the sulfate-reducing bacterium *Desulfovibrio fructosovorans*, house a dinuclear active site deeply buried within the protein and composed of one nickel and one iron atoms [2–4]. The crystal structures also show the linear arrangement within the small subunit of three iron–sulfur clusters, one [3Fe–4S] surrounded by two [4Fe–4S], located between the active site and the protein surface. The three iron–sulfur centers form the intramolecular electron pathway allowing H_2 production/oxidation [5–7]. These enzymes also use channels to guide the diffusion of the gaseous substrate H_2 to and from the deeply buried active site [8–10]. Hydrogenases are inhibited by several small molecules including O_2, CO, acetylene and NO [9,11–15]. While inhibition of hydrogenases by O_2 or CO has been widely studied, much less is known about the mechanism of NO inhibition. The very first work on cell-free extracts of *Proteus vulgaris* established that NO is a powerful inhibitor of hydrogenases [16]. It was also concluded that this inhibition can be partly reversed at low NO concentration (below 0.2 μM), unlike at higher concentration. Later, Tibelius et al. have shown that cell-extracts from *Azospririllum brasilense* are irreversibly inhibited by NO [17]. The first study on purified hydrogenases was carried out by studying the effect of NO on the H^+/D^+ exchange activity of *Desulfovibrio* hydrogenases [18]. The authors concluded that these enzymes are severely and irreversibly inhibited at low NO concentrations and that the iron–sulfur clusters are not affected. The effect of NO on the H_2-uptake activity of the soluble hydrogenase from *Cupriavidus metallidurans*[2] [19] and of the membrane-bound hydrogenase from *Azotobacter vinelandii* [20], was interpreted as a complex mechanism including a reversible and an irreversible processes. The authors interpreted this inhibition pattern as an interaction of NO with different sites of the enzyme, including the FeS centers. More recently, the study of inhibition of hydrogenases by NO regained interest because NO can contaminate the syngas in the biomass-producing biological systems in which hydrogenases play a main role [21]. In this work, NO was interpreted as a non-competitive inhibitor interacting with several sites of hydrogenases, including the FeS clusters [21]. So far, NO inhibition has been studied only by measuring hydrogenase activities and, to our knowledge, no molecular study has been undertaken to unambiguously identify the sites of interaction within the protein.

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NO is known to interact irreversibly with FeS clusters, resulting in thiolate-ligated dinitrosyl iron complexes (DNIC) [22–24] characterized by an axial EPR signal at $g = 2.03$ [25]. Mononuclear DNIC is not the only species composed by the $Fe(NO)_2$ unit [26]. Other nitrosylated species have been reported and characterized in the literature: the dimeric Roussin's red ester (RRE) and a nitrosylated [4Fe–3S] cluster known as Roussin's black salt. Oxidized DNIC and reduced RRE (rRRE) can be detected by EPR spectroscopy as a $S = 1/2$ species [27].

In the present work, we combine protein film electrochemistry and EPR spectroscopy to study the molecular mechanism of NO interaction with the purified periplasmic NiFe hydrogenase from *D. fructosovorans* and to determine where NO binds within the protein. We show that NO interacts in a complex manner with the NiFe active-site and also induces irreversible damage of the electron transfer iron–sulfur centers chain.

2. Methods

2.1. Purification of hydrogenase, conditions of growth, strains and activity measurement

The strep-tagged WT and mutants (V74M, V74W) of the *D. fructosovorans* hydrogenase were constructed using the bacterial strains, plasmids, growth conditions, site-directed mutagenesis strategy and the enzyme purification protocol as described in references [9,12]. The H_2-oxidation activity was assayed with methyl viologen as electron acceptor as previously described [9].

2.2. Nitric oxide solution

All experiments (except PFV, see below) were performed in 50 mM HEPES at pH 8 with NO-saturated solution by using a NO gas cylinder (Messer). The concentration of NO in the NO-saturated solution was estimated to 1.9 mM at 25 °C, based on solubility data [28]. The gas was passed through an alkaline trap (aqueous anaerobic solution of KOH at 5 M) before being bubbled in the buffer solution in order to prevent formation of NO_x species and especially nitrite [29]. Despite these precautions, we could not avoid nitrite contamination in our NO-saturated solutions as shown by the UV spectrum of NO-saturated solutions [30]. We thus performed specific control experiments in which EPR spectra of hydrogenase were recorded in the presence of 1 mM nitrite. We also recorded chronoamperograms of hydrogenase films exposed to nitrite up to 3 mM. We observed that nitrite used in this range of concentration does not affect enzymatic activity and does not change EPR spectra of hydrogenase.

2.3. Protein film voltammetry

The electrochemical equipment has been fully described in reference [31] (supplementary information therein). The electrochemical experiments were carried out in a "mixed buffer" consisting of MES, HEPES, sodium acetate, TAPS, and CHES (5 mM of each component), 1 mM EDTA, and 0.1 M NaCl as supporting electrolyte, titrated to pH 4 or 7 using concentrated HCl or NaOH. Before preparing an enzyme film, the rotating disc pyrolytic graphite edge (PGE) electrode was polished with aqueous alumina slurry (Buehler, 1 μm) and sonicated thoroughly for 10–20 s. Protein films were prepared by painting the electrode with about 0.5 μL of a stock solution of enzyme (about 0.4 mg/mL) of *D. fructosovorans* NiFe hydrogenase in the mixed-buffer at pH 7. The electrode could then be rinsed and transferred to a fresh solution with little loss in electroactive coverage over time. All electrochemical experiments were carried out in anaerobic glove boxes filled with N_2 ($pO_2 < 1$ ppm, JACOMEX gloveboxes).

We analyzed the electrochemical data using open source, in-house programs described in reference [32] and available for download on our web site at www.qsoas.org. The currents were normalized and corrected for film loss as described in [32].

2.4. Preparation of samples for EPR spectroscopy

All EPR studies have been performed using fresh preparations of about 40–50 μM NiFe hydrogenase in 50 mM HEPES at pH 8. Enzyme samples were carefully degassed with argon to minimize O_2 contamination and subsequently transferred into an anaerobic glovebox.

Two types of samples were prepared:

- "*Open*": Aliquots of protein sample were divided into several EPR tubes, then various volumes of NO-saturated buffer were added to each protein sample to get different ratios of NO over enzyme (from 1 to 25 equivalents). Small volumes of buffer solution were also added to obtain EPR samples with a final volume of 160 μL and a final enzyme concentration of 40 μM. After addition of NO-saturated buffer, the EPR tubes were sealed by a septum. The enzyme was incubated with the NO solution in the sealed EPR tube for a time ranging between 5 and 60 min, prior freezing in cold ethanol (−114 °C) in the glovebox. EPR tubes were subsequently kept in liquid nitrogen outside the glovebox. It is important to note that, despite the fact that the EPR tubes were sealed, the NO present in the protein solution can fully escape in the gas phase above (4/5 of total volume of the EPR tube), hence the designation of "open" preparation.
- "*Closed*": The above experiments were reproduced by incubating the enzyme with NO in a gas-tight micro syringe to restrict the volume of the gas phase above the protein solution. In these conditions, NO exchange between gas and liquid phases was limited. We prepared two samples to obtain 10 and 25 equivalents NO/enzyme. Protein solution and NO-saturated buffer were sucked up in the gas-tight micro syringe to get enough solution for filling three EPR tubes. We mixed the contents with a little stir bar previously introduced inside the syringe. After various incubation times with NO, 160 μL of protein solution were then transferred into an EPR tube and frozen.

The reduced state of the two types of samples above was obtained by adding small volumes (between 10 and 20 μL) of a degassed dithionite solution (100 mM in 50 mM HEPES pH 8) to the protein directly in the EPR tube (160 μL of protein sample at 40 μM). Dithionite-reduced samples were then incubated for a few minutes under a N_2 atmosphere prior to freezing.

2.5. EPR spectroscopy

X-band EPR spectra were recorded with a Bruker ELEXSYS E500 spectrometer fitted with a microwave cavity and an ESR-900 helium flow cryostat (Oxford Instruments). Spin quantifications were made by using an external $[CuEDTA]^{2-}$ standard (1 mM Cu(II), 10 mM EDTA, 20 mM TRIS pH 8) and by comparing the double integral of the spectra to that of the standard under non-saturating conditions.

3. Results

3.1. Kinetics of nitric oxide inhibition of hydrogenase studied by protein film voltammetry

To study the kinetics of inhibition of hydrogenase by NO under turnover conditions, we used protein film voltammetry [33] and the methods that we introduced before to characterize the reaction of hydrogenases with gaseous molecules like CO or O_2 [11,12,31,34]. In these experiments, a small amount of enzyme (less than a pmol) is adsorbed onto an electrode immersed in a solution that contains H_2. If the potential of the electrode is high enough (greater than the H^+/H_2 reduction potential), the enzyme oxidizes H_2. Turnover results in a