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Interaction of HydSL hydrogenase from *Thiocapsa roseopersicina* with cyanide leads to destruction of iron-sulfur clusters

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ABSTRACT

The effects of cyanide on enzymatic activity and absorption spectra in the visible and mid-IR (2150–1850 cm^{-1}) regions were characterized for purified HydSL hydrogenase from the purple sulfur bacterium *Thiocapsa (T.) roseopersicina* BBS. Prolonged incubation (over hours) of *T. roseopersicina* hydrogenase with exogenous cyanide was shown to result in an irreversible loss of activity of the enzyme in both the oxidized (as isolated) and H_2-reduced states. The frequency position of the active site CO and CN^- ligand stretching bands in the Fourier transform infrared (FTIR) spectrum of the oxidized form of hydrogenase was not influenced by cyanide treatment. The 410-nm absorption band characteristic of hydrogenase iron-sulfur clusters showed a bleaching concomitantly with cyanide inactivation. A new band at 2038 cm^{-1} was present in the FTIR spectrum of the cyanide-inactivated preparation, which band is assignable to ferrocyanide as a possible product of a destructive interaction of hydrogenase with cyanide. The results are interpreted in terms of a slow destruction of iron-sulfur clusters of hydrogenase in the presence of cyanide accompanied by a release of iron ions in the form of ferrocyanide into the surrounding solution. Such a slow and irreversible cyanide-dependent inactivation seems to be complementary to a recently described rapid, reversible inhibitory reaction of cyanide with the active site of hydrogenases [S.V. Hexter, M.-W. Chung, K.A. Vincent, F.A. Armstrong, J. Am. Chem. Soc. 136 (2014) 10470–10477].

1. Introduction

Hydrogenases are metalloenzymes that belong to the class of oxidoreductases and catalyze the activation of molecular hydrogen:

$2H^+ + 2e^- \leftrightarrow H_2$.

To date, more than three dozen hydrogenases from microorganisms of different taxonomic groups have been isolated and obtained in a homogeneous state; for some of them three-dimensional crystal structures were determined [1]. There are three types of hydrogenases differing in the content of metals in their active site: [NiFe]-hydrogenases containing a bimetallic iron-nickel center; [FeFe]-hydrogenases having a diatomic iron center; Fe-hydrogenases that do not contain metals but include a Fe-containing cofactor [2]. For most [NiFe]-hydrogenases, the bimetallic center was shown to be covalently bound to the protein by four cysteine residues, two of which form a connecting bridge between Ni and Fe, and the other two coordinate Ni. The Fe ion is associated with diatomic ligands, one CO ligand and two CN^- ligands. Depending on the redox state of the enzyme, a non-protein ligand may be present between the metal ions [3].

To investigate mechanisms of function of enzymes, and hydrogenases among them, various inhibitors are widely used [4]. In particular, much attention has been paid to studying the inhibition of the catalytic function of [NiFe]-hydrogenases by carbon monoxide. It was shown that exogenous CO acts directly on the active site, binding as a ligand to the Ni ion and competing with H_2, thus reducing the catalytic activity of the enzyme [5,6]. An effective inhibitor of metalloenzymes is also cyanide [7]. The irreversible inhibitory effects of cyanide on the activity of membrane-associated and purified hydrogenase from *Azotobacter (A.) vinelandii* has been described in Ref. [8]. Based on spectrophotometric assay methods, it was shown [8] that exogenous cyanide caused an irreversible inactivation of the hydrogenase in the oxidized state but had no effect on the enzyme in the reduced state. As the most probable explanation of the inactivating effect, it was suggested that cyanide binds to one or more metal-containing centers of the enzyme [8]. Recent electrochemical, EPR, and attenuated total reflectance infrared (ATR-IR) measurements [9] have showed that cyanide is capable of a rapid reaction with isolated Hyd1 and Hyd2 [NiFe]-hydrogenases from *Escherichia (E.) coli* under oxidizing conditions, inhibiting electrocatalytic oxidation of hydrogen. A mechanism of

Abbreviations: ATR-IR, attenuated total reflectance infrared; FeS clusters, iron-sulfur hydrogenase clusters; FTIR, Fourier transform infrared

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inhibition was proposed [9] according to which cyanide acts as a strong promoter of the formation of the inactive oxidized state Ni-B at the active site of both enzymes. It was shown that cyanide-induced inhibition was easily reversed upon the reductive activation of Ni-B [9] that could account for the fact that cyanide was an inhibitor only under oxidizing conditions [8,9].

Studies of the structure, catalytic properties and resistance against inactivating factors of the membrane-bound HydSL hydrogenase from the photosynthetic purple sulfur bacterium *Thiocapsa (T.) roseopersicina* BBS are of considerable interest both in terms of clarifying the mechanism of the functioning of hydrogenases and for practical purposes. When immobilized on the electrode, this thermostable hydrogenase is considered as a promising hydrogen-activating catalyst for the development of new types of fuel cells and hydrogen sensors based on direct bioelectrocatalysis [10]. From the comparison with hydrogenases of other organisms, this hydrogenase from *T. roseopersicina* was referred to [NiFe]-hydrogenases [11]. The protein of *T. roseopersicina* hydrogenase was shown to consist of two subunits with a molecular weight of 64 and 34 kDa, respectively. The catalytic active site is deeply buried in the larger subunit, and the smaller subunit contains three iron-sulfur clusters, which form a transport system for intramolecular electron transfer between the active site and the surface of the enzyme [11]. The active site of the hydrogenase contains a Ni ion [12]. An X-ray crystal structure of *T. roseopersicina* hydrogenase is not available. Recently, an *in silico* model of the spatial structure of hydrogenase from *T. roseopersicina* has been proposed, based on the analysis of the amino acid sequence of the protein and the crystallographic structure known for the [NiFe]-hydrogenase from *Allochromatium (A.) vinosum* [13]. The model suggests a high degree of similarity between the three-dimensional structures of the two enzymes.

Earlier, it was reported that *T. roseopersicina* HydSL hydrogenase in the oxidized state showed almost no inactivation (about 2%) when treated with exogenous potassium cyanide over 1 h [14]. In view of the apparent discrepancy between this result and the data obtained for the two other microorganisms whose hydrogenases showed a significant inhibitory effect of cyanide under oxidizing conditions [8,9], we reexamine here the initial observation [14]. We show that prolonged incubation of the purified hydrogenase from *T. roseopersicina* with potassium cyanide results in a considerable loss of functional activity, independent of the redox state of the enzyme (oxidized or reduced). Based on absorption measurements in the visible and mid IR spectral regions, we provide evidence that the observed slow and irreversible cyanide inactivation of *T. roseopersicina* hydrogenase is due to a destruction of iron-sulfur clusters, rather than a consequence of interactions between cyanide and the active site of the enzyme.

2. Materials and methods

2.1. Purification of hydrogenase

Cells of the purple sulfur bacterium *T. roseopersicina* BBS were grown under anaerobic photoheterotrophic conditions on a modified Pfennig medium [15] in the presence of 0.2% sodium acetate. Cells were separated from the culture liquid at the end of the exponential growth phase. To obtain cell extracts, 200 g of the cell paste was resuspended in 20 mM potassium phosphate buffer (pH 7.0) in a ratio of 1:1, and cells were destroyed by treatment with acetone and ultrasonication as described previously [16]. Purification of the hydrogenase was performed under aerobic conditions using the fractionation of cell extracts with ammonium sulfate and sequential liquid chromatography on columns with phenylsepharose CL-4B (Pharmacia) and DEAE-cellulose DE52 (Whatman) [17]. The final stage of purification of the hydrogenase preparation was carried out by preparative electrophoresis in 7% polyacrylamide gel as described earlier [18]. If necessary, hydrogenase samples were concentrated using centrifugal microconcentrators Microcon-10 (10 kDa cuttoff membranes; Amicon).

2.2. Hydrogenase activity assay

Hydrogenase activity was determined spectrophotometrically by measuring methyl viologen (Sigma) reduction with hydrogen in stoppered glass cuvettes purged with H_2 [16]. The reaction mixture (total volume of 2 ml) contained 50 mM Tris-HCl buffer (pH 9.0), 4 mM methyl viologen and 0.01–0.10 μM hydrogenase. Traces (5–10 μl) of the 20 mM sodium dithionite (Fluka) solution prepared under anaerobic conditions were added to facilitate activation of the enzyme under 1 atm H_2 during 5–10 min. All assays were performed at 30 °C. To calculate the activity, the extinction coefficient of the reduced methyl viologen at 600 nm (ε_{600} = 13 $mM^{-1}{\cdot}cm^{-1}$) was used [19]; the hydrogenase activity was expressed as μmol of H_2/min per 1 mg of protein.

2.3. Reaction with cyanide

For experiments, a freshly prepared 1 M KCN (Sigma) solution in 1 mM KOH was used to minimize the inhibitor hydrolysis. Hydrogenase preparations were incubated with KCN in 20 mM potassium phosphate buffer (pH 7.0) at room temperature in a stoppered glass cuvette, aerobically or in H_2 or CO atmosphere. To follow the reaction between cyanide and the enzyme, aliquots of the incubation mixture were removed at various times during incubation and injected into the assay cuvette to determine for remaining hydrogenase activity as described above. With this procedure, changes in H_2 oxidation activity caused by an irreversible inactivation of the enzyme could be evaluated. In order to remove the inhibitor from the reaction media after incubation, samples were subjected to 3 times cycles of diluting with the buffer and subsequent concentrating on a 10 kDa cuttoff membrane using centrifugal microconcentrators Microcon-10 (Amicon).

2.4. Optical spectroscopy

Fourier transform infrared (FTIR) spectra were recorded at room temperature on a Bruker IFS66v/S spectrophotometer equipped with an MCT detector (D313/6) and a KBr beam splitter. Some measurements were performed using a Bruker Equinox 55 spectrometer equipped with a DTGS detector and a KBr beam splitter. The spectral resolution was 2 cm^{-1}. Samples of hydrogenase (~1 mM) in 10 mM or 50 mM potassium phosphate buffer (pH 7.0) were placed in a liquid cell with CaF_2 windows (19 mm in diameter) separated by a 50 μm spacer. The enzyme reduction was performed under H_2 atmosphere for 20 h. In experiments with cyanide, hydrogenase was incubated with KCN added to a final concentration of 10 or 50 mM (see figures' legends). For each IR spectrum of hydrogenase 500 scans were averaged. Spectra were corrected for the water background by subtraction of the potassium phosphate buffer spectrum measured under similar conditions. Further correction of the baseline in the spectra was performed in a manual mode using the OPUS software (Bruker).

Electronic absorption spectra were measured with a Shimadzu 1800 spectrophotometer.

3. Results and discussion

3.1. FTIR spectroscopy of T. roseopersicina hydrogenase

There is a significant number of publications (for a review, see Refs. [3,4]), which show that a characteristic feature of metal-containing hydrogenases is the presence at their active site of diatomic ligands, carbon monoxide and cyanide. In the absence of crystallographic data, FTIR spectroscopy makes it possible to identify and study of stretching bands of ligands associated with metal ions in hydrogenases, and provides valuable information for analyzing the redox states of enzymes [20–27]. With the intention of employing FTIR measurements to examine possible interactions of cyanide with *T. roseopersicina*