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Characterization and *in vitro* interaction study of a [NiFe] hydrogenase large subunit from the hyperthermophilic archaeon *Thermococcus kodakarensis* KOD1

Daisuke Sasaki [a], Satoshi Watanabe [a], Tamotsu Kanai [b], Haruyuki Atomi [b], Tadayuki Imanaka [c], Kunio Miki [a,*]

[a] Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
[b] Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan
[c] Department of Biotechnology, College of Life Sciences, Ritsumeikan University, Noji-Higashi, Kusatsu, Shiga 525-8577, Japan

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ABSTRACT

The large subunit of the [NiFe] hydrogenases harbors a $NiFe(CN)_2(CO)$ cluster. Maturation proteins HypA, B, C, D, E, and F are required for the NiFe cluster biosynthesis. While the maturation machinery has been hitherto studied intensively, little is known about interactions between the Hyp proteins and the large subunit of the [NiFe] hydrogenase. In this study, we have purified and characterized the cytosolic [NiFe] hydrogenase large subunit HyhL from *Thermococcus kodakarensis* (*Tk*-HyhL). *Tk*-HyhL exists in equilibrium between monomeric and dimeric forms. *In vitro* interaction analyses showed that *Tk*-HyhL monomer forms a tight complex with *Tk*-HypA and weakly interacts with *Tk*-HypC. The expected ternary complex formation was not detected. These observations reflect a diversity in the mechanism of Ni insertion in [NiFe] hydrogenase maturation depending on the organism.

1. Introduction

Hydrogenases, which are widely distributed in bacteria, archaea, and some eukaryotes, catalyze the reversible formation and consumption of molecular hydrogen and contribute to energy metabolism [1]. The core structure of the [NiFe] hydrogenase consists of a large and a small subunit [2]. The large subunit carries a $NiFe(CN)_2(CO)$ cluster at the active site [3–5]. The NiFe cluster is not formed spontaneously. The biosynthesis/maturation of the [NiFe] hydrogenase proceeds through a stepwise process performed by HypA, HypB, HypC, HypD, HypE, and HypF as well as specific endopeptidases [6–8]. At present, the overall maturation process is proposed as follows; First, HypC, HypD, HypE and HypF catalyze the synthesis and incorporation of a Fe atom ligated with diatomic CN and CO [9–13]. Second, the Ni atom is inserted into the large subunit. HypA and HypB have been shown to play a major role in this step [14–19]. Finally, specific endopeptidases such as HycI cleave off the C-terminal tail of the large subunit to form an active "mature" enzyme followed by binding of the small subunit [20].

The functions of the Hyp proteins have been widely investigated in *Escherichia coli* and other bacteria [6]. Furthermore, we have performed structural and functional analyses of the Hyp proteins from the hyperthermophilic archaeon *Thermococcus kodakarensis* KOD1 [21–26]. These results have provided significant insights into the functional roles of each Hyp protein and the interactions among these Hyp proteins during the maturation process. However, little is known about how the Hyp proteins interact with the large subunit of the [NiFe] hydrogenase.

In this study, we have purified and characterized the cytosolic [NiFe] hydrogenase large subunit, HyhL from *T. kodakarensis* (*Tk*-HyhL). SEC analyses of mixtures containing *Tk*-HyhL and Hyp proteins indicate a tight complex formation between *Tk*-HyhL and *Tk*-HypA, and weak interaction between *Tk*-HyhL and *Tk*-HypC.

2. Materials and methods

2.1. Cloning, expression, and purification of the large subunit Tk-HyhL and maturation proteins Tk-HypA and Tk-HypC

The gene fragment encoding *Tk*-HyhL (TK2069, 428 a.a.) was amplified by PCR using the genomic DNA of *T. kodakarensis* KOD1 [21] with the forward primer 5′-gg**tctag**aaataattttgtttaactttaagaaggagatatacatatgaagaacgtttatctcccgatcaccg-3′ (*XbaI* site is shown in

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ICP-AES, inductively coupled plasma-atomic emission spectrometry; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SEC, size exclusion chromatography; Tris, tris (hydroxymethyl)aminomethane.

* Corresponding author. Fax: +81 75 753 4032.
E-mail address: miki@kuchem.kyoto-u.ac.jp (K. Miki).

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bold) and the reverse primer 5′-cgc**ggatcc**gcaagcctcgccacgtgcac-3′ (*Bam*HI site is shown in bold). The amplified gene was inserted into pUC19 and, after confirming the sequences of the DNA fragments, the fragments were inserted into *Xba*I/*Bam*HI sites of pET21a(+) (hyhL_pET21a(+)) (Novagen). The *E. coli* Rosetta2(DE3)pLysS cells were transformed with hyhL_pET21a(+). The transformants were plated on Luria–Bertani (LB) agar plates containing 34 μg/mL of chloramphenicol and 100 μg/mL of ampicillin. Single colonies were separately picked and pre-cultured overnight at 37 °C. One percent (v/v) of pre-culture was inoculated into LB medium containing the same antibiotics as the LB agar plate. Expression of the recombinant protein was induced with 0.1 mM IPTG when cell density reached 0.4 at OD_{600} and was further incubated for 6 h at 37 °C. The harvested cell pellet was suspended in 50 mM Tris–HCl pH 8.0, 1 mM DTT, and the suspension was sonicated on ice with an output energy of 50 W for a total period of 150 s. The supernatant after centrifugation (30,000*g*, 30 min at 4 °C) was subjected to heat treatment at 80 °C for 10 min. The supernatant after centrifugation (30,000*g*, 30 min at 4 °C) was filtrated with a 0.22 μm pore filter (Millipore) and applied onto an anion exchange column (ResourceQ 6 mL, GE healthcare) equilibrated with 50 mM Tris–HCl pH 8.0, 1 mM DTT. Proteins were eluted with a 0–0.2 M linear gradient of NaCl. The eluted sample was concentrated and applied onto a size exclusion column (Superdex 200 10/300 GL 24 mL, GE healthcare) equilibrated with 20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM DTT at a flow rate of 0.5 mL/min. All chromatography procedures were performed at room temperature using ÄKTA explorer system (GE healthcare) under aerobic conditions.

Molecular mass of the purified sample was estimated with size exclusion chromatography (SEC) by comparing its retention volume to those of marker proteins (GE healthcare), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease A (17.3 kDa), and aprotinin (6.5 kDa). The K_{av} value is defined by the equation $K_{av} = (V_e - V_o)/(V_c - V_o)$, where V_e, V_o, and V_c are the elution, column void, and geometric column volumes, respectively. Oligomeric states were also confirmed by non-denaturing native-PAGE analysis. The quantitative and qualitative metal analyses of the sample were performed by ICP-AES using Optima 4300DV (PerkinElmer Inc., Waltham, MA, USA). UV–visible absorption spectrum was measured with NanoDrop 1000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

To obtain metal free samples, EDTA was added to a final concentration of 10 mM in 50 mM Tris–HCl pH 8.0, 1 mM DTT prior to the cell sonication. EDTA was removed from the supernatant after heat-treatment by buffer exchange, and an anion exchange column chromatography was performed. $(NH_4)_2SO_4$ was added to a concentration of 1.5 M and the sample was applied onto a hydrophobic interaction column (Resource ISO 6 mL, GE healthcare) equilibrated with the same buffer. Proteins were eluted by a linear 1.5–0.3 M gradient of $(NH_4)_2SO_4$ at a flow rate of 6.0 mL/min. Size exclusion column chromatography was subsequently performed.

Expressions and purifications of *Tk*-HypA and *Tk*-HypC were performed as previously reported [24,26].

2.2. Interaction analysis between the large subunit and maturation protein

SEC analysis was carried out by using a size exclusion column (Superdex 200 10/300 GL 24 mL, GE healthcare) equilibrated with 20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM DTT. Protein mixture solutions (200 μl) containing the indicated amounts of *Tk*-HyhL and Hyp proteins were prepared by mixing the proteins and incubating overnight at room temperature. As for the mixtures containing three proteins, three kinds of the mixtures were prepared. Mixture I was prepared by adding *Tk*-HyhL to the mixture of *Tk*-HypA and *Tk*-HypC. Mixture II was prepared by adding *Tk*-HypC to the mixture of *Tk*-HypA and *Tk*-HyhL. Mixture III was prepared by adding *Tk*-HypA to the mixture of *Tk*-HypC and *Tk*-HyhL. These protein mixtures were subjected to heat-treatment at 85 °C for 15 min after overnight incubation. Complex formation was also confirmed by non-denaturing native-PAGE analysis.

3. Results

3.1. Characterization of the large subunit Tk-HyhL

T. kodakarensis possesses three distinct [NiFe] hydrogenase homologs on its genome; one encodes the cytosolic hydrogenase Hyh, another encodes the membrane-bound hydrogenase Mbh, whereas the third encodes a complex (Mbx) which does not exhibit hydrogenase activity and whole function is still not clear [22,23]. In this study, the cytosolic [NiFe] hydrogenase large subunit gene, the *Tk-hyhL* gene was cloned and the recombinant protein was successfully obtained using an *E. coli* expression system. An elution profile of the SEC of *Tk*-HyhL showed two major peaks (the *bold line* in Fig. 1A). The estimated molecular masses of *Tk*-HyhL proteins from these peaks are ~49 and ~94 kDa, which correspond to a monomer (48.3 kDa) and a dimer (96.6 kDa), respectively. When fractions containing only the monomer or dimer were individually reapplied to SEC, both monomeric and dimeric states were observed (data not shown). These results clearly show that *Tk*-HyhL is in slow equilibrium between monomeric and dimeric states. This monomer–dimer equilibrium was also observed by non-denaturing native-PAGE analysis (Fig. 1B). The equilibrium was partially shifted to the monomer when the sample was subjected to heat-treatment at 85 °C for 15 min and analyzed by SEC (data not shown).

The purified sample was brown in color. An ICP-AES analysis showed that the sample contained 0.23 Fe atoms per molecule of *Tk*-HyhL, along with a small amount of Zn and Ca atoms. No Ni atoms were detected. A UV–visible absorption spectrum of the *Tk*-HyhL solution showed broad absorption bands in 300–700 nm (the *bold line* in Fig. 1C). Monitoring at the wavelengths of 330 and 450 nm during SEC showed that both monomer and dimer contained Fe atoms (data not shown). An EDTA treated sample showed no absorption bands in the UV spectrum (the *broken line* in Fig. 1C), indicating that the Fe atom was completely removed from the sample. The EDTA treated sample also existed in the monomer–dimer equilibrium (data not shown).

3.2. In vitro interaction analysis between Tk-HyhL and Tk-HypA or Tk-HypC by SEC

When SEC was performed with a mixture of *Tk*-HypA and *Tk*-HyhL, the two proteins co-eluted as a new single peak (the *arrow* in Fig. 2A), indicating tight complex formation between these two proteins. The HyhL–HypA complex appeared at an elution volume of ~15.2 mL corresponding to an estimated molecular mass of ~63 kDa, which is consistent with the sum of the calculated molecular masses of *Tk*-HypA (15.7 kDa) and *Tk*-HyhL monomer (*Tk*-HyhLm) (48.3 kDa). These results indicate that *Tk*-HyhL forms a 1:1 binary complex with *Tk*-HypA. The EDTA treated sample of *Tk*-HyhL also formed a 1:1 complex with *Tk*-HypA (data not shown).

When a mixture of *Tk*-HypC and *Tk*-HyhL at an equal molar ratio was applied to the column, the peak height of *Tk*-HypC subtly decreased (the *gray arrow* in Fig. 2B) and the elution peak of *Tk*-HyhLm slightly shifted to a higher molecular mass (the *black arrow* in Fig. 2B). When the mixtures were prepared at a 2–3-fold molar excess of *Tk*-HypC, the peak height of *Tk*-HyhLm in SEC