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Selective interaction of Hpn-like protein with nickel, zinc and bismuth in vitro and in cells by FRET

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ABSTRACT

Hpn-like (Hpnl) is a unique histidine- and glutamine-rich protein found only in *Helicobacter pylori* and plays a role on nickel homeostasis. We constructed the fluorescent sensor proteins *CY*Hpnl and *CY*Hpnl_1-48 (C-terminal glutamine-rich region truncated) using enhanced cyan and yellow fluorescent proteins (eCFP and eYFP) as the donor–acceptor pair to monitor the interactions of Hpnl with metal ions and to elucidate the role of conserved Glu-rich sequence in Hpnl by fluorescence resonance energy transfer (FRET). *CY*Hpnl and *CY*Hpnl_1-48 exhibited largest responses towards Ni(II) and Zn(II) over other metals studied and the binding of Bi(III) to *CY*Hpnl was observed in the presence of an excess amount of Bi(III) ions ($K_d = 115 \pm 4.8$ μM). Moreover, both *CY*Hpnl and *CY*Hpnl_1-48 showed positive FRET responses towards the binding to Ni(II) and Zn(II) in *Escherichia coli* cells overexpressing *CY*Hpnl and *CY*Hpnl_1-48, whereas a decrease in FRET upon Bi(III)-binding in *E. coli* cells overexpressing the latter. Our study provides clear evidence on Hpnl binding to nickel in cells, and intracellular interaction of Hpnl with Bi(III) could disrupt the protein function, thus probably contributing to the efficacy of Bi(III) drugs against *H. pylori*.

1. Introduction

Helicobacter pylori resides in acidic gastric environment and infects more than 50% of the world population, leading to peptic ulcer and even gastric cancer [1,2]. Two nickel-containing metalloenzymes, [NiFe] hydrogenase [3,4] and urease [5–7], are essential for the survival and colonization of *H. pylori* under an extremely low pH condition [8]. The intracellular level of nickel is thus tightly regulated in uptake, trafficking, storage and release, accounting for its necessity and toxicity [7,9,10]. Histidine-rich Hpn and histidine- and glutamine-rich Hpn-like (Hpnl) proteins have been found to serve as nickel reservoirs in *H. pylori*, which supply the metal for the maturation of hydrogenase and urease (Tables S4–S5) [6,11–14]. In scarce nickel conditions, wild-type *H. pylori* colonizes more efficiently in mice than both *hpn* and *hpnl* gene deleted *H. pylori*, pinpointing the importance of Hpn and Hpnl in *H. pylori* colonization under its physiological condition [15]. Although the role of Hpnl has been proposed, its interaction with metal ions in the actual cellular environment has not been clearly demonstrated.

Bismuth-based medications, such as colloidal bismuth subcitrate (DeNol®, Lizhudele®), bismuth subsalicylate (Pepto-Bismol®) and ranitidine bismuth citrate (Pylorid®), have been used for the treatment of microbe-causing gastrointestinal infections for decades, in particular to eradicate *H. pylori* infection when combined with antibiotics [16–20]. Proteins, especially enzymes, are believed to be the therapeutic targets although the molecular mechanism of bismuth drugs is not fully understood and the intracellular monitoring of drug actions is still lacking [5,15,17].

Fluorescence resonance energy transfer (FRET) has been widely used to examine the reaction kinetics through monitoring the conformational changes of biomolecules upon their interactions with corresponding substrates [21–23]. Fluorescent proteins frequently serve as FRET donor–acceptor pairs given that they can be genetically-encoded to the proteins of interest, and the fusion proteins are expressed for fluorescence studies inside cells [24,25]. Moreover, genetically-encoded FRET sensors can also be used to elucidate the concentration of metal ions in cells, owing to their abilities of accurately targeting various subcellular organelles [24,26,27].

In this work, we engineered a fluorescent sensor protein *CY*Hpnl to examine the interaction of Hpnl towards various metal ions by FRET, utilizing eCFP and eYFP as the FRET donor–acceptor pair. To investigate the role of highly conserved C-terminal glutamine-rich sequence, a mutant of *CY*Hpnl (*CY*Hpnl_1-48) was constructed and a similar study was carried out. We further explored the metal-binding properties of Hpnl by overexpressing *CY*Hpnl or *CY*Hpnl_1-48 in bacterial cells. The metal selectivity of Hpnl in vitro and in cells is compared and the effect of bismuth drug is discussed.

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2. Experimental

2.1. Materials

Primers were synthesized by Life Technologies. All chemicals were purchased from Sigma-Aldrich or USB, and were used without further purification.

2.2. Construction of pET32a–eCFP–Hpnl–eYFP and C-terminal glutamine-deleted mutant pET32a–eCFP–Hpnl_NoCterQ–eYFP expression vectors

The full-length *ecfp* and *eyfp* genes were PCR-amplified from plasmids pECFP-Nuc and pEYFP-C1 (Clontech Laboratories) by primer pairs (CFP-For/CFP-Rev and YFP-For/YFP-Rev). The *hpnl* gene was amplified from *H. pylori* 11637 genomic DNA using primer Hpnl-For/Hpnl-Rev (Table S1). After digestion by corresponding restriction endonucleases (New England Biolabs), the PCR products *ecfp* and *eyfp* were successively inserted into pET32a vector to obtain the plasmid pET32a–eCFP–eYFP and hpnl gene was inserted between *ecfp* and *eyfp* genes to obtain the plasmid pET–eCFP–Hpnl–eYFP (named pET-*CY*Hpnl hereafter). Linkers with a sequence of Gly-Ser-Gly-Ser were inserted among the genes of *ecfp*, *hpnl* and *eyfp* to enhance the flexibility and solubility of the sensors.

For the C-terminal glutamine-deleted mutant pET32a–eCFP–Hpnl_1-48–eYFP (named pET-*CY*Hpnl_1-48 hereafter), *hpnl* gene was amplified with primer pair (Hpnl-For/Hpnl-NoCQ-Rev) and the mutant *hpnl* gene was inserted into the pET32a–eCFP–eYFP plasmid. Plasmid pET28a–eCFP–eYFP (named pET-*CY*FP hereafter) was also constructed to produce a fluorescent sensor protein consisting of only the flexible linker (Gly-Ser-Gly-Ser) between the genes of eCFP–eYFP FRET donor–acceptor pair for control experiments.

2.3. Expression and purification of CYHpnl and CYHpnl_1-48

Plasmids pET-*CY*Hpnl and pET-*CY*Hpnl_1-48 were transformed into *Escherichia coli* BL21(DE3). The overnight culture was subcultured into fresh Luria-Bertani (LB) medium with 1:100 dilution supplemented with 100 μg/ml ampicillin and was grown at 37 °C until OD_{600} reached 0.6. Protein expression was induced overnight at 25 °C in the presence of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Bacterial pellets were harvested by centrifugation and resuspended in buffer A (20 mM Tris–HCl, pH 7.6, 500 mM NaCl, and 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP)) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF).

Cells were lysed by sonication, then the lysates was centrifuged at 10,000 *g* at 4 °C for 30 min and the supernatant was applied to HisTrap HP column (GE Healthcare). The column was washed with buffer A in the presence of an increasing amount of imidazole (from 20 to 150 mM) and the protein was eluted with buffer A containing 200 mM imidazole. Fractions were collected and were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. S1). The peak fraction was further purified by Resource Q column (GE Healthcare) with a linear gradient of NaCl from 0 to 500 mM and the peak fractions were pooled and dialyzed against Buffer B (10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.8, 150 mM $(NH_4)_2SO_4$) three times at 4 °C for 6 h for subsequent use. Protein concentration was determined by BCA Protein Assay Kit (Novagen). ICP-MS analysis (Agilent 7500a spectrometer) was performed to ensure that the protein remained in its apo-form prior to fluorescence analysis.

2.4. In vitro FRET analysis

Full fluorescence emission spectra were obtained on a Hitachi F7000 Fluorescence Spectrophotometer using 1 cm x 1 cm quartz cuvette. *CY*Hpnl (250 nM in Buffer B) was excited at 433 nm with 1000 W xenon lamp source at the excitation and emission slit width of 5 nm. The photomultiplier voltage was set to be 650 V and emission spectra were scanned from 450 to 650 nm at a speed of 40 nm per second.

Fluorescence experiments were carried out on a Beckman Coulter DTX880 Multimode Detector, using the excitation filter 405/30 nm and emission filter 465/30 nm and 535/30 nm. Intensity ratio of the two emission signals (emission ratio = 535/30:465/30) was used to quantify the FRET changes. Metal stock solutions were prepared from $NiSO_4$, $ZnSO_4$, $CoSO_4$, $MnSO_4$, $MgSO_4$, $CuSO_4$ and $Bi(NO_3)_3$ (in glycerol). Molar equivalents of various metal ions were added to 400 nM of *CY*Hpnl or *CY*Hpnl_1-48 or *CY*FP (as a control) in Buffer B. The changes in FRET emission ratio (emission at filters 535/30:465/30) were calculated by averaging the results of at least three replicates.

2.5. Metal-binding capacities of CYHpnl

The dissociation constants (K_d) for Ni(II)- and Zn(II)-*CY*Hpnl were determined by equilibrium dialysis. Apo-*CY*Hpnl (20 μM) solution was filled into home-made dialysis tubes (3 kDa cutoff) and was dialyzed against Buffer B supplemented with a series of concentrations of Ni(II) and Zn(II) ions overnight at 4 °C. ICP-MS was employed to determine the metal concentrations in and outside the dialysis tubes while nonlinear fitting with Hill equation in GraphPad Prism 5 ($Y = B_{max} * X \wedge h / (K_d \wedge h + X \wedge h)$, where B_{max} is the maximum specific binding, h is the Hill coefficient and K_d is the dissociation constant) was used to calculate the dissociation constant (K_d) for Ni(II)- and Zn(II)-binding to *CY*Hpnl after normalization with protein concentration.

To determine the K_d of Bi(III) to *CY*Hpnl, nonlinear fitting with Hill equation in GraphPad Prism 5 ($Y = B_{max} * X \wedge h / (K_d \wedge h + X \wedge h)$) was employed to analyze the FRET results with titration of Bi to *CY*Hpnl. To determine the stoichiometries of metal binding to *CY*Hpnl, *CY*Hpnl (20 μM) was incubated with six molar equivalents of Ni(II), Co(II), Cu(II), Zn(II) and Bi(III) and excess metal ions were removed by buffer exchange prior to ICP-MS analysis.

2.6. FRET analysis in E. coli cells

Plasmid pET-*CY*Hpnl or pET-*CY*Hpnl_1-48 was transformed into BL21(DE3) *E. coli* cells. An overnight culture was inoculated into fresh LB medium in the presence of 100 μg/ml of ampicillin. Cells were harvested by centrifugation at 4000 rpm when OD_{600} reached 0.6 and were subsequently washed three times with freshly prepared MOPS minimal medium [28] containing 100 μg/ml of ampicillin. Cells were then grown at 37 °C in MOPS minimal medium for 30 min after washing for recovery. Protein expression was induced at 25 °C for at least 16 h with the use of 0.2 mM IPTG. The OD_{600} of bacterial culture was measured and subsequently diluted to the OD_{600} of 0.4. Various concentrations of Ni(II), Zn(II) and Bi(III) ions (as colloidal bismuth subcitrate), ranged from 0 to 200 μM, were then added to the bacterial cell culture medium for a four-hour incubation at 25 °C prior to fluorescence measurement. Samples were subjected to Beckman Coulter DTX880 Multimode Detector for fluorescence measurement with the same settings as in in vitro FRET experiments. *CY*FP-overexpressed *E. coli* cells were also subjected to FRET analysis after a four-hour incubation with Ni(II), Zn(II), Mg(II) and Bi(III) (0–200 μM) as a control.

3. Results

3.1. Detection of metal binding by FRET and in vitro metal selectivity and affinity of CYHpnl

The strategy for the design and fabrication of the fluorescent sensor proteins are illustrated in Fig. 1B. We first investigated the occurrence of FRET between eCFP and eYFP due to the conformational change in the target Hpnl protein by fluorescence spectroscopy using purified *CY*Hpnl. Addition of Ni(II) ions to *CY*Hpnl led to a decrease in the eCFP fluorescence at 476 nm and an increase in the eYFP fluorescence (525 nm),