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Conserved cysteine residues are necessary for nickel-induced allosteric regulation of the metalloregulatory protein YqjI (NfeR) in *E. coli*

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ABSTRACT

Transition metal homeostasis is necessary to sustain life. First row transition metals act as cofactors within the cell, performing vital functions ranging from DNA repair to respiration. However, intracellular metal concentrations exceeding physiological requirements may be toxic. In *E. coli*, the YqjH flavoprotein is thought to play a role in iron homeostasis. YqjH is transcriptionally regulated by the ferric uptake regulator and a newly discovered regulator encoded by *yqjI*. The apo-form of YqjI is a transcriptional repressor of both the *yqjH* and *yqjI* genes. YqjI repressor function is disrupted upon binding of nickel. The YqjI N-terminus is homologous to nickel-binding proteins, implicating this region as a nickel-binding domain. Based on function, *yqjI* and *yqjH* should be renamed Ni-responsive Fe-uptake regulator (*nfeR*) and Ni-responsive Fe-uptake flavoprotein (*nfeF*), respectively. X-ray Absorption Spectroscopy was employed to characterize the nickel binding site(s) within YqjI. Putative nickel binding ligands were targeted by site-directed mutagenesis and resulting variants were analyzed in vivo for repressor function. Isothermal titration calorimetry and competitive binding assays were used to further quantify nickel interactions with wild-type YqjI and its mutant derivatives. Results indicate plasticity in the nickel binding domain of YqjI. Residues C42 and C43 were found to be required for in vivo response of YqjI to nickel stress, though these residues are not required for in vitro nickel binding. We propose that YqjI may contain a vicinal disulfide bond between C42 and C43 that is important for nickel-responsive allosteric interactions between YqjI domains.

1. Introduction

Effective homeostatic management of iron, a biologically-abundant transition metal, is essential for most organisms. Iron is a cofactor for proteins with roles in metabolism (ie. cellular respiration and photosynthesis), dioxygen storage and transport, and redox sensing and signaling mechanisms. Iron primarily exists in ferrous (Fe(II)) and ferric (Fe(III)) oxidation states. Fe(III), the predominant oxidation state in aerobic environments, has limited solubility at neutral pH making iron acquisition difficult for many organisms. Under conditions of limited iron availability, microbial organisms can produce iron chelators known as siderophores to facilitate metal solubility and transport into the cell [1]. However, excess Fe(II) in the cell can lead to redox cycling with hydrogen peroxide to generate radical species that can oxidize lipids, DNA, and amino acids [2].

Due to the delicate balance required to maintain necessary iron levels without accruing a damaging excess, the genes governing iron uptake, use, and export are meticulously regulated at the transcriptional and post-transcriptional levels. The ferric uptake regulator (Fur) controls expression of > 100 genes that are involved in iron usage or homeostasis in the Gram-negative bacterium *Escherichia coli* [3]. Under iron-rich conditions the Fe^{2+}-Fur dimer represses target genes, but this repression is relieved under iron-deficient conditions, allowing for increased expression of genes involved in iron homeostasis [4,5]. The gene *yqjH* is one member of the Fur regulon. The YqjH enzyme catalyzes the flavin adenine dinucleotide (FAD)-dependent reduction of ferric iron to the ferrous state. Previous studies have established that YqjH catalyzes iron-release from a variety of chelators, such as ferric dicitrate and ferric triscatecholate, using an NADPH dependent reduction mechanism [6]. This work suggested an in vivo role for YqjH in the reduction of ferric iron from multiple siderophores [6]. Based on these studies we propose that the hypothetical gene name *yqjH* be replaced in

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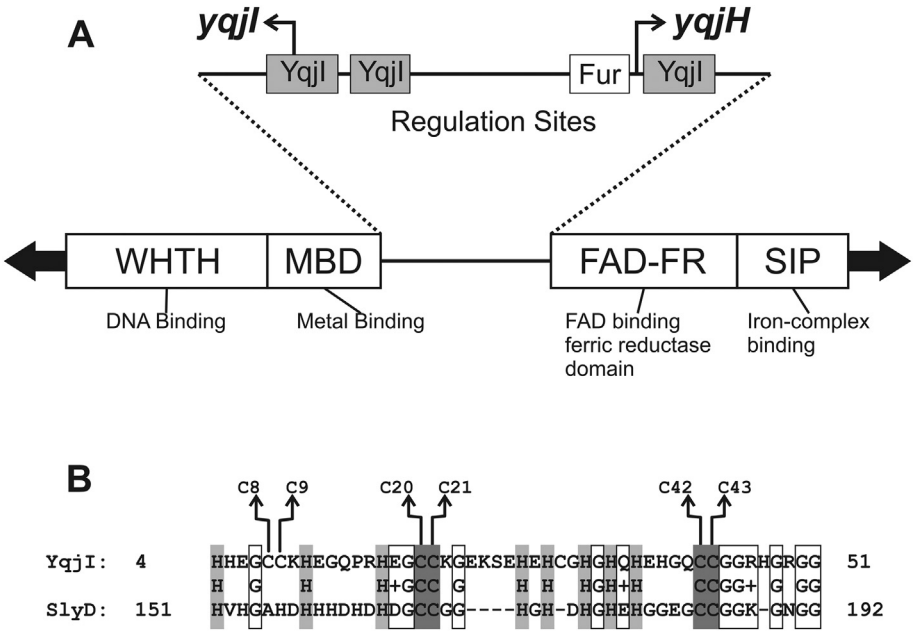

Fig. 1. A. Intergenic region between *yqjH* and *yqjI* genes as well their genetic coding regions. Binding sites for YqjI and Fur in the promoters of both genes are indicated in the top image. **B**. Metal binding N-terminus of YqjI showing homology to the nickel-binding region of SlyD. Light grey boxes indicate conserved His residues, dark grey boxes indicate conserved Cys residues, and open boxes indicate other conserved residues.

the literature with Nickel-responsive Fe-uptake Flavoprotein, abbreviated as *nfeF*.

Intriguingly, transcription of *yqjH* is also strongly repressed independently of Fur by the YqjI protein. The *yqjI* gene is encoded adjacent to the 5′-end of the *yqjH* gene, but is transcribed in the opposite direction, as shown in Fig. 1A. YqjI residues 57 to 207 are homologous to the PadR family of transcriptional regulators, suggesting a possible function for YqjI as a transcriptional repressor [7–9]. Earlier work established that YqjI binding to sites within the promoter regions of both *yqjI* and *yqjH* results in transcriptional repression of both genes (Fig. 1A) [10]. Wang et al. determined that three similar, palindromic binding sites for YqjI are present within the promoter regions. Two of these sites are near the *yqjI* transcriptional start site while the third is proximal to the start site for *yqjH* [11]. By suppressing *yqjH* gene expression, YqjI indirectly prevents iron reduction, and thus iron release from siderophores [6].

The N-terminus of the YqjI protein includes a ~52 residue-long region consisting of 7 cysteine, 12 histidine, and 7 glutamate residues. This region lacks any predicted secondary structure and may be disordered (Fig. 1B) [10]. Sequence analysis reveals that this region is similar to a nickel-binding region within the peptidyl-prolyl cis-trans isomerase SlyD also present in *E. coli*. The binding of nickel ions to SlyD regulates its isomerase activity [12]. In addition, the YqjI N-terminus also resembles the nickel-binding region within the metal exporter RcnA [13]. Previously, it was shown that addition of Ni(II) or Fe(II) could disrupt YqjI DNA-binding in vitro [10]. Further investigation revealed that in vivo repression of *yqjH* and *yqjI* transcription by YqjI is abolished upon addition of high levels of nickel but addition of iron had no effect [10]. Together these results suggest that nickel binding to the YqjI N-terminus may negatively regulate YqjI DNA-binding activity such that transcription of the *yqjH* ferric reductase is increased under high nickel conditions. Based on these studies we propose that the hypothetical gene name *yqjI* be replaced in the literature with Nickel-responsive Fe-uptake Regulator, abbreviated as *nfeR*.

In the current study, we present the first detailed characterization of nickel-binding to YqjI (NfeR). Simulations of the Extended X-ray Absorption Fine Structure (EXAFS) region of the nickel K-edge XAS spectra were used to identify the types of ligands responsible for YqjI nickel-binding, and their relevant metal-ligand bond lengths. Site-directed mutagenesis coupled with in vivo gene regulation studies were used to determine the functional relevance of the candidate ligands within the YqjI N-terminus. Then competitive nickel-binding assays and chemical analyses were performed to probe the YqjI-nickel interactions. The results indicate that nickel-dependent regulation of YqjI requires several conserved Cys residues in the N-terminus. Furthermore, the conformational flexibility and ligand-rich nature of the YqjI N-terminus may allow for multiple nickel-binding modes.

2. Materials and methods

2.1. Strains and plasmid preparation

YqjI and its mutant derivatives were expressed from the pET21a vector (Novagen) as previously described [10]. Mutations to YqjI cysteines were constructed following the QuikChange II site-directed mutagenesis protocol (Agilent). All mutations were confirmed by sequencing and mutated plasmids were transformed into BL21(DE3) cells for expression and purification of YqjI. All strains and primers can be found in Tables S1 and S2.

2.2. ApoYqjI and NiYqjI expression and purification

YqjI was expressed from a pET21a vector in *E. coli* BL21(DE3) cells as previously described but with some modifications [10]. Briefly, cells grown overnight in LB with 100 mg/L Ampicillin (Amp) were diluted 1:100 in fresh LB Amp (100 mg/L) and grown at 37 °C with 200 rpm shaking to an OD_{600} of 0.7 to 0.8. For expression of apoYqjI, the cells were induced with 10 μM isopropyl β-D-1-thiogalactopyranoside for 24 h at 25 °C after which they were harvested via centrifugation at 7460 ×*g* for 10 min and stored at −80 °C until use. Cells were resuspended in heparin binding buffer consisting of 20 mM HEPES, 200 mM NaCl, 5% glycerol, 10 mM β-mercaptoethanol (βME), pH = 7.5 with 1 mM phenylmethylsulfonyl fluoride added. After resuspension, the cells were lysed via sonication for two 2-min intervals of one second on and one second off at 50% amplitude on a Branson digital sonifier 450. Lysed cells were treated with 1% streptomycin sulfate and centrifuged for 40 min at 31000 ×*g*. Purification was performed following the previously established protocol using the cation exchange (Hitrap), nickel (Histrap), and gel filtration chromatography (Superdex200) columns in sequence [11]. The monomeric form of YqjI was isolated by gel filtration (Fig. S1A). Purity of final YqjI fractions was determined using SDS-PAGE and the protein was concentrated via