Supplementary Material (ESI) for Chemical Communications

SUPPORTING MATERIAL

A Structural and Functional Homolog Supports a General Role for Frataxin in Cellular Iron Chemistry

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MATERIALS and METHODS

General Chemicals. HEPES, Tris, DTT, $Na_2S \cdot 10H_2O$, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, NaCl and G-25 were purchased from Sigma-Aldrich (St. Louis, MO).

Cloning and Expression of *Bacillus subtilis* YdhG. *Bacillus subtilis* genomic DNA was obtained from the American Type Culture Collection (ATCC no. 23857D). 100 ng DNA, 100 ng of each primer, 2.5 units of cloned *Pfu*Turbo DNA polymerase, *Pfu*Turbo buffer, and 0.2 mM of each dNTP were used to amplify *Bs YdhG* (locus tag BSU05750) in *Bacillus subtilis* via PCR. Primers were designed as follows: 5'-GACGCCATGGAG TGGCATCATTTTATTTTAG-3' and 5'-TATTCTCGAGT TCCGCCAAAAAGTGGA-3', where the underlined regions denote the *Nco*I and *Xho*I restriction sites, respectively. The thermocycle used was identical to that described in the *Pfu*Turbo DNA polymerase manual (Stratagene). PCR products were digested with *Nco*I and *Xho*I and ligated to double-digested vector pET28-b(+) by T4 DNA ligase. Cloning into pET28-b(+) resulted in the production of the gene for C-terminal His-tagged *B. subtilis* YdhG. Cloning results were confirmed by nucleotide sequencing, and *E. coli* BL21(DE3) was used for

protein expression. A 10 ml Luria-Bertani (LB) culture with 30 mg/L kanamycin was grown overnight as a starter culture, which was then used to inoculate 1 L LB medium and grown to an OD_{600} of ~ 0.6 prior to induction with 0.3 mM isopropyl-1-thio-α-D-galactopyranoside (IPTG). Cells were pelleted 5 h after induction and stored at –80 °C for future use.

Purification of *Bs* YdhG. C-terminal His tag *Bs* YdhG Protein was mostly expressed as inclusion bodies, so in-column purification and refolding was performed on Ni-NTA column. Purification and Refolding of *Bs* YdhG. Protein was mostly expressed in the inclusion body, so in-column purification and refolding was performed(*1*). Cell pellets were resuspended in five volumes of 50 mM Tris-HCl, pH 7.4, with 1 mM PMSF, and lysed by sonication followed by the addition of 8M urea and stirring for 30 minutes to solubilize the protein in inclusion body. Insoluble material was removed by centrifugation at 15,000 rpm, 4 °C for 30 minutes. The clear lysate was applied to a Ni-NTA column equilibrated with binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 500 mM NaCl, 8M urea). The column was then washed with five column volumes of binding buffer + 10 mM imidazole and five volumes of Buffer A (20 mM Tris-HCl, pH 7.9, 100 mM NaCl). 10 volumes of Buffer A + 0.1% Triton-X was then applied to the column to stimulate the refolding and then washed by 10 volumes of Buffer A + 5 mM β-cyclodextran slowly. β-cyclodextran was washed out by 10 volumes of Buffer A. Refolded protein (*2*)was eluted by Buffer A + 500 mM imidazole and concentrated by Amicon to about 5 mL. Purified YdhG protein was dialyzed to 50 mM Hepes, pH 7.5, 100 mM NaCl. Purified *Bs* YdhG, as judged from SDS-PAGE, was dialyzed into 50 mM Hepes, pH 7.5, 100 mM NaCl.

Light Scattering from *Bs* YdhG with and without added Fe^{2+}.

Dynamic light scattering (DLS) was performed on a Malvern Instruments Zetasizer Nano-ZS. To a 60 μM solution of *Bs* YdhG in 50 mM Hepes, 100 mM NaCl, 2 mM dithionite, and 1 mM TCEP at

pH=7.5 was added 0.5 to 5 equivalents of Fe^{2+} anaerobically from a stock solution of 1.5 mM $Fe(NH_4)_2(SO_4)_2$ in 50 mM Hepes, 100 mM NaCl, also at pH=7.5. All samples were filtered through a 0.20 µm filter before size measurements were made. Data analysis was performed using Zetasizer Software and Origin.

Quantitation of Iron Binding to Bs YdhG by Isothermal Titration Calorimetry (ITC). ITC measurements of ferrous ion binding to Bs YdhG were carried out at 25 C on a MicroCal ultrasensitive titration calorimeter. A 100 µM Bs YdhG solution in 50 mM Hepes pH 7.5, 100 mM NaCl with 5 mM dithionite and 1 mM TCEP was rigorously degassed and loaded into the cell. A 1.5 mM ferrous solution in the same buffer was titrated into the sample in 10 µL aliquots over a period of 24 s with 6 min intervals between injections. To avoid the oxidation of Fe^{2+}, the buffer was purged by Argon gas before transferred to a vacumned vial to dissolve $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and the solution was quickly loaded to the syringe. Data was collected automatically and subsequently analyzed with an Origin Software package provided by MicroCal.

Quantitation of *Tm* IscU Binding to *Bs* YdhG by ITC. ITC measurements of *Tm* IscU binding to *Bs* YdhG were carried out at 25 C on a MicroCal ultrasensitive titration calorimeter. A solution of 30 µM *Bs* YdhG in 50 mM Hepes pH 7.5, 100 mM NaCl, 2 mM dithionite and 150 µM Fe^{2+} was rigorously degassed and loaded into the cell. A 300 µM *Tm* IscU solution in the same buffer was titrated into the sample in 10µL aliquots over a period of 24 s at 6 min intervals. The titration was repeated under the same conditions in the absence of Fe^{2+} in either the cell or the syringe. Data were collected automatically and subsequently analyzed with Origin Software package provided by MicroCal. Control experiments, following titration of titrant into buffer, or buffer into titrant were also performed and used to correct for background heat changes.

Quantitation of D46A Human ISU Binding to *Bs* YdhG by ITC. ITC measurements of D46A human ISU binding to *Bs* YdhG were carried out at 25 ºC on a MicroCal ultrasensitive titration calorimeter. A 20 μM *Bs* YdhG solution in 50 mM Hepes pH 7.5, 300 mM NaCl with 2 mM dithionite and 100 μM Fe^{2+} was rigorously degassed and loaded into the cell. A 250 μM *human* ISU solution in the same buffer was titrated into the sample in 10 μL aliquots over a period of 24 s at 6 min intervals. The titration was repeated under the same conditions in the absence of Fe^{2+} in either the cell or the syringe. Data were collected automatically and subsequently analyzed with Origin Software package provided by MicroCal. Control experiments, following titration of titrant into buffer, or buffer into titrant were used to correct for background heat changes.

Quantitation of Iron Binding to *Bs* YdhG by UV-vis and fluorescence. A solution of 20 μM *Bs* YdhG in 50 mM Hepes, pH 7.5, 100mM NaCl, and 5 mM dithionite was titrated by aliquots of 100 μM or 500 μM ferrous stock solutions in the same buffer. The absorbance at 278 nM and the tryptophan fluorescence (excitation 280 nm, emission 350 nm) were each monitored.

Tm IscU Iron-Sulfur Cluster Reassembly Mediated by Bs YdhG. A solution of 100 μM Tm IscU, 40 μM Bs YdhG was mixed with 10 mM DTT and degassed. The reaction was initiated by the addition of 200 μM ferrous ion and 2.5 mM sulfide and monitored by the increase of absorbance at 456 nm. The reaction was repeated without Bs YdhG. Control background responses were subtracted from both sets of data for the reaction without Tm IscU or Bs YdhG

Supplementary Figures

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Human Frataxin  56 -SSNQRGLNQIWNVKKQSVYLMNLRKSGTLGHPGSLDETTYERLAEETLDSLAEFFEDLADKPYTFEDYDVSFGSGVLTV 134
Yeast Frataxin  52 VESSTDG-----QVVPQEVLNLPLEK--------------YHEEADDYLDHLLDSLEELSEAHPDCIP-DVELSHGVMTL 111
Tt NQO15         1 MSASSER-----ELYEAWVELLSWMR-------------EYAQAKGVRFEKEADFPDFIYR-----MERPYDLPTTIMTA  57
Bs ydhG          1 ----MEWHHFILGGNNMDVFSEYLAG-------------IADPFHRERTEEVLTWIKNKYPNLHTEIKWNQPMFTDHGTF  63
                                       *                                 :     .              :     *

Human Frataxin 135 KLGGDLGTYVINKQTPNKQIWLSSP-SSGPKRYDWTGKNWVYSHDGVSLHELLAAELTKALKTKLDLSSLAYSGKDA 210
Yeast Frataxin 112 EIP-AFGTYVINKQPPNKQIWLASP-LSGPNRFDLLNGEWVSLRNGTKLTDILTEEVEKAISKSQ------------ 174
Tt NQO15        58 SLSDGLGEPFLLADVSPRHAKLKRIGLRLPRAHIHLHAHYEPGKGLVTGKIPLTKERFFALADRAREALAFA----- 129
Bs ydhG         64 IIGFSVSKKHLAVAPEKVTIAHVED-DIVKAGYDYTEQLIRIPWNGPVDYTLLEKMIEFNILDKADCSTFWRK---- 135
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Figure S1. Multiple sequence alignment of human frataxin, yeast frataxin, *Thermus thermophilus* (Tt) Nqo15 and *Bacillus subtilis* (Bs) YdhG. Human and yeast proteins exclude the mitochondrial targeting sequences. For *Bs* YdhG the group of 5 structurally conserved carboxylate residues are highlighted in yellow, along with the human residues, and in green in the structure shown in Figure 1 in the main text.

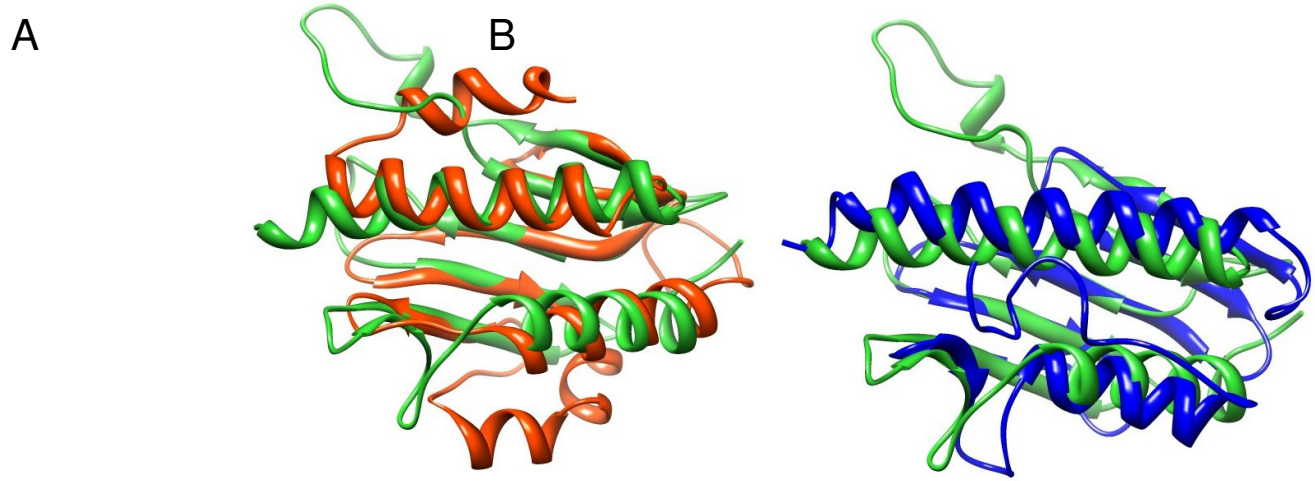

Figure S2. Structure comparison of (**A**) *T. thermophilus* Nqo15 and *Bs* YdhG; (**B**)human frataxin and *T. thermophilus* Nqo15. Color coding: blue, human frataxin; green, *T. thermophilus* Nqo15; orange, *Bs* YdhG.

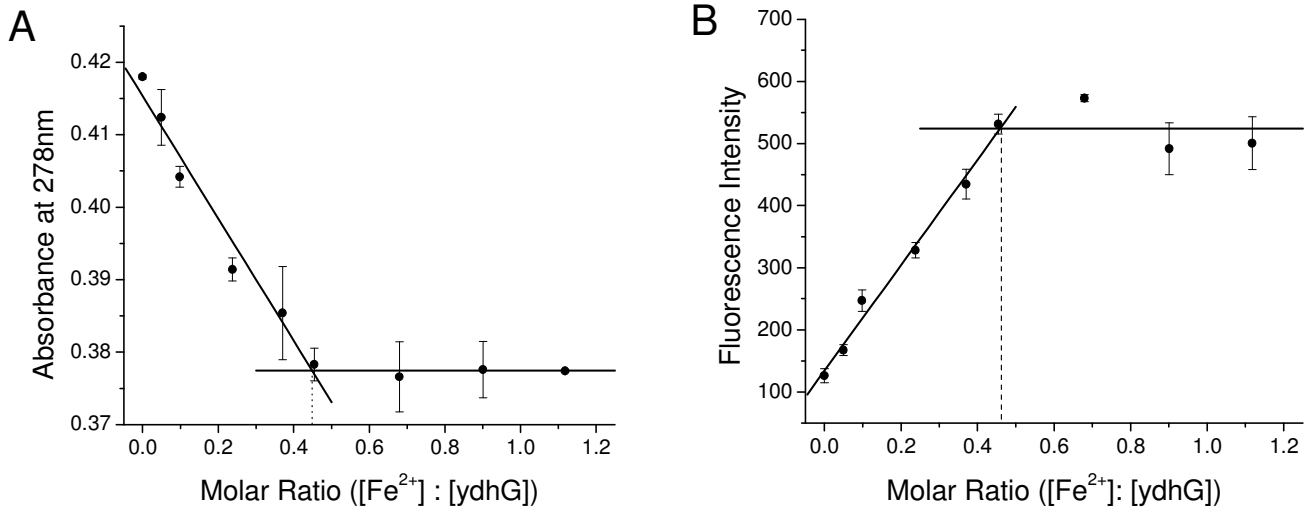

Figure S3. Binding of Fe^{2+} was monitored by the change of absorbance at 278 nm (**A**); and the change of intrinsic tryptophan fluorescence (**B**). Absorbance and fluorescence data were fit to two linear domains and the stoichiometry determined to be 0.45 and 0.46, respectively.

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Bs IscU        MSFNANLDTLYRQVIMDHYKNPRNKGVLN---DSIVVDMN-NPTCGDRIRLTMKLDG-DI 55
Tm IscU        ----MVFKMMYSEAILDYANSKKFRGKLD---DATVIEEGKNISCGDEITLYLKVED-GV 52
Human ISU      -MVLIDMSVDLSTQVVDHYENPRNVGSLDKTSKNVGTGLVGAPACGDVMKLQIQVDEKGK 59
E. Coli IscU   --------MAYSEKVIDHYENPRNVGSFDNNDENVGSGMVGAPACGDVMKLQIKVNDEGI 52
                           ::*: :. :  * ::   .          :*** : * ::::  .

Bs IscU        VEDAKFEGEGCSISMASASMMTQAIKGKDIETALSMSKIFSDMMQGKEYDDSIDLGDIEA 115
Tm IscU        VKDAKFEGMGCVISQASASLMLERIIGERVEEIFSLIEEAEKMSRGENFDEGK-LKNVTL 111
Human ISU      IVDARFKTFGCGSAIASSSLATEWVKGK-------------------TVEEALTIKNTDI 100
E. Coli IscU   IEDARFKTYGCGSAIASSSLVTEWVKGK-------------------SLDEAQAIKNTDI 93
               : **:*:  **  : **:*:  : : *:                     ::.  : :

Bs IscU        LQGVSKFPARIKCATLSWKALEKGVAKEEGGN---- 147
Tm IscU        MSDIKNYPARVKCFILAWKTLKEALKKISRP----- 142
Human ISU      AKELCLPPVKLHCS-------KSVLFPAEEKTQLSP 129
E. Coli IscU   AEELELPPVKIHCSILAEDAIKAAIADYKSKREAK- 128
                . :   *.:::*        :  :   .
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Figure S4. Multiple sequence alignment of *Bs* IscU, *Tm* IscU, *E. coli* IscU and *Hs* ISU, showing the 19-amino acid insertion in *Bs* IscU and *Tm* IscU.

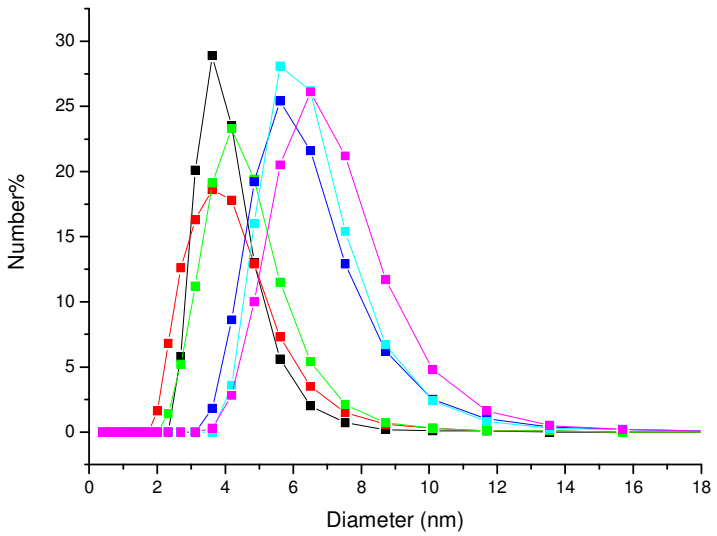

Figure S5. Light scattering profile for *Bs* YdhG with the addition of Fe^{2+}. Black: YdhG only; Red: YdhG with 0.5 equiv. Fe^{2+}; Green: YdhG with 1 equiv. Fe^{2+}; Blue: YdhG with 2 equiv. Fe^{2+}; Cyan: YdhG with 4 equiv. Fe^{2+}; Magenta: YdhG with 5 equiv. Fe^{2+}.

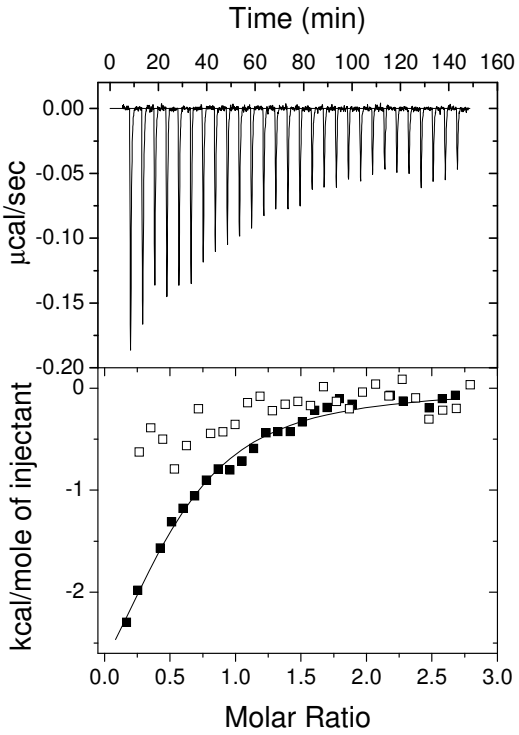

Figure S6. ITC measurement of human ISU binding to *Bs* YdhG. A 250 µM D46A human ISU solution was titrated into 20 µM *Bs* YdhG in 10 µL aliquots with 100 µM Fe^{2+} in both the cell and syringe. Data were fit to a one site binding model and the resulting parameters determined as n = 0.52 ± 0.06, K_D = 6.7 ± 0.5 µM, ΔH = -42 ± 6 kcal·mol^{-1}, ΔS = 10 cal·K^{-1}·mol^{-1}. Control data showing titration of D46A human ISU to Bs YdhG in the absence of Fe^{2+} are shown by open squares. The greater noise compared to Figure 2B reflects the smaller overall enthalpy change for this binding event.

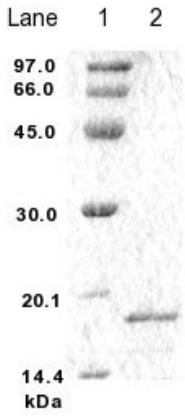

Figure S7. SDS-polyacrylamide gel (12%) for the purified *Bs* YdhG. *Lane 1*, molecular weight markers (97, 66, 45, 30, 20.1, 14.4 kDa from the top); *lane 2*, Isolated *Bs* YdhG following the purification protocol described earlier.