Supporting Information for

Characterization of a [4Fe-4S]-Dependent LarE Sulfur Insertase that Facilitates Nickel-Pincer Nucleotide Cofactor Biosynthesis in *Thermotoga maritima*

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Running Title: [4Fe-4S]-dependent sulfur-transferase LarE
**Figure S1. Sequence comparison of LarE<sub>rm</sub> to selected other LarE sequences.** The sequences were chosen from those identified by BLAST using the non-redundant GenBank coding sequence translations with E-scores below e-73. Full species names for the genera shown are: *Thermotoga maritima*, *Ignisphaera aggregans*, *Candidatus Hydrothermae bacterium*, *Thermococci archaeon*, *Deltaproteobacteria bacterium*, *Methanotrichaceae archaeon*, *Methanoseta sp. PtaU1.Bin112*, *Methanothrix soehngenii GP6*, *Methanophagales archaeon*, *Methanoregula formicica*, *Thermoplasmata archaeon*, *Euryarchaeota archaeon*, *Actinomyctetia bacterium*, *Armatimonadetes bacterium*, *Anoxybacter fermentans*, *Zhaonella formicivorans*, *Calderihabitans maritimus*, *Carboxythermus hydrogenoformans*, *Thermoanaerobacteraceae bacterium*, *Chloroflexi bacterium*, *Geospirillum subterraneus*, *Firmicutes bacterium*, *Halocella sp. SP3-1*, *Dictyoglomi bacterium*, *Dictyoglomus thermophilum*, *Clostridium homopropionicum*, *Synergistales bacterium 54_24*, *Acetomicrobium sp. S15* (DSM 107314). The sequence alignment was performed using Multalin (43). Identities are shown in red font. An SGGXDS motif associated with PP-loop pyrophosphatase family members is indicated by the green bar. Three cysteines associated with a CXXC-C motif are shown by the two blue segments. A fourth cysteine present in three sequences is shown by the yellow star.
Figure S2. Mass spectrometric analysis reveals a lack of change for LarE<sub>Tm</sub> during P2TMN synthesis. The His<sub>6</sub>-tagged LarE subunit lacking its amino-terminal methionine residue remains unchanged in size when comparing samples that were incubated with P2CMN, 20 mM MgCl<sub>2</sub>, and 2 mM ATP for 0, 30, and 60 min. The small peak at m/z 31,186 likely represents His<sub>6</sub>-tagged LarE in a mixed disulfide linkage with βME.

Figure S3: SEC MALS analysis of His<sub>6</sub>-tagged LarE<sub>Tm</sub>. The results indicate a dimeric quaternary structure.
Figure S4. Comparison of cysteine desulfurase sequences and structures from *E. coli* (IscS\textsubscript{Ec}) and one of two related proteins in *T. maritima* (IscS\textsubscript{Tm}). (a) Sequence alignment. (b) Crystal structure of IscS\textsubscript{Ec} (PDB: 3LVK) including the bound pyridoxal phosphate (space fill depiction) and showing the cysteine residue (stick view) that forms a persulfide. (c) Homology model of IscS\textsubscript{Tm} depicting the conserved cysteine residue (stick view).
### Table S1

**Native mass spectrometry peak list for the 12+ monomeric species of LarE<sub>Tm</sub>**

| LarE<sub>Tm</sub> Monomer + Species | Theoretical m/z | Actual (peak) m/z | Difference (m/z) |
|-----------------------------------|----------------|------------------|-----------------|
| Na<sup>++</sup>                   | 2634.8         | 2635.1           | 0.3             |
| Fe                                | 2637.5         | 2637.5           | 0.0             |
| [Fe-S]                            | 2640.2         | 2640.3           | 0.1             |
| [Fe-2S]                           | 2642.8         | 2642.8           | 0.0             |
| [2Fe-S]                           | 2644.8         | 2645.7           | 0.9             |
| [2Fe-2S]                          | 2647.5         | 2648.3           | 0.8             |
| [2Fe-3S]                          | 2650.2         | 2650.9           | 0.7             |
| [3Fe-3S]                          | 2654.8         | 2654.0           | 0.8             |
| [3Fe-4S]                          | 2657.5         | 2657.3           | 0.2             |
| [4Fe-4S]                          | 2662.2         | 2661.9           | 0.3             |

<sup>a</sup> For each species shown, the numbers within the brackets represent the total number of iron and sulfur atoms associated with the LarE<sub>Tm</sub> monomer subunit and do not necessarily equate to the type of bound iron-sulfur cluster. The masses tabulated include the theoretical m/z, the actual (peak) m/z, and the calculated difference, with values near zero providing confidence in the iron and sulfur content assignments.

### Table S2

**Native mass spectrometry peak list for the 15+ dimeric species of LarE<sub>Tm</sub>**

15+ Dimer (63,476 Daltons)

| LarE<sub>Tm</sub> Dimer + Species | Theoretical m/z | Actual (peak) m/z | Difference (m/z) |
|-----------------------------------|----------------|------------------|-----------------|
| [3Fe-4S][3Fe-4S]                  | 4256.2         | 4257.4           | 1.2             |
| [4Fe-4S][4Fe-4S]                  | 4263.7         | 4262.6           | 1.1             |
| [4Fe-5S][4Fe-5S]                  | 4265.8         | 4264.8           | 1.0             |
| [4Fe-5S][5Fe-5S]                  | 4271.7         | 4273.1           | 1.4             |
| [5Fe-5S][5Fe-5S]                  | 4275.4         | 4275.5           | 0.1             |

<sup>a</sup> For each species shown, the numbers within the brackets represent the total number of iron and sulfur atoms associated with each LarE<sub>Tm</sub> subunit in the dimer and do not necessarily equate to the type of bound iron-sulfur cluster. The masses tabulated include the theoretical m/z, the actual (peak) m/z, and the calculated difference, with values near zero providing confidence in the iron and sulfur content assignments.
References:
CORPET, F. (1988) Multiple sequence alignment with hierarchical clustering. *Nucl. Acids Res.* 16 (22), 10881-10890