Supporting information for article:

A three-domain copper-nitrite reductase with a unique sensing loop

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Table S1  Cupredoxins with reported crystal structures that resembles the C-terminal extra domain of TsNirK.

| Protein     | PDB  | Microorganism                 | Loop Cys-Met | % Identity |
|-------------|------|--------------------------------|---------------|------------|
| Domain III-TsNirK |      | *Thermus scotoductus* SA-01 | CSIHPYM       | -          |
| Amicyanin (*PdAmi*) | 1MDA | *Paracoccus denitrificans*     | CTPHPFM       | 31         |
| Pseudoazurin | 3TU6 | *Sinorhizobium meliloti* 2011 | CAPHVGM       | 31         |
| Amicyanin (*PvAmi*) | 1ID2 | *Paracoccus versutus*         | CTPHPFM       | 30         |
| Pseudoazurin | 1BQK | *Achromobacter cycloclastes*  | CTPHYGM       | 25         |
| Plastocyanin | 1BXU | *Synechococcus sp.*           | CEPHRGAGM     | 25         |
| Plastocyanin | 1JXD | *Synechocystis PCC6803*        | CEPHRGAGM     | 21         |
| Azurin       | 1RKR | *Alcaligenes xylosoxidans*    | CSFPGHFALM    | 19         |
Table S2  Geometric parameters of TsNirK T1Cu centres and its comparison with other T1Cu.

| Protein                      | T1 Centres |  |  |  |  |  |  |  |  |  |
|-----------------------------|------------|---|---|---|---|---|---|---|---|---|
|                             | Ts         | Hd| Gk| Ax| Af| Cs| Ste| Ami|
|                             | TsNirK     | Nir| Nir| Nir| Nir| Nir| Ste| Ami|

| Distances (Å)               | C          | N  | C  | N  | -  | -  | -  | -  | -  |
|-----------------------------|------------|---|---|---|---|---|---|---|---|
| Cu–His₁N⁶¹                  | 2.02(0.01) | 2.05(0.01) | 2.14 | 2.12 | 2.07 | 2.02 | 2.08 | 1.96 | 2.04 |
| Cu–CysS⁷                    | 2.27(0.02) | 2.18(0.04) | 2.13 | 2.23 | 2.13 | 2.20 | 2.22 | 2.18 | 2.13 |
| Cu–His₂N⁶¹                  | 2.04(0.02) | 2.04(0.01) | 2.10 | 2.11 | 2.03 | 2.03 | 2.07 | 2.04 | 2.13 |
| Cu–MetS⁵                    | 3.02(0.04) | -  | 2.55 | 2.90 | 2.61 | 2.45 | 2.45 | -  | 2.84 |
| Cu–GlnOε¹                   | -  | 2.03(0.01) | -  | -  | -  | -  | -  | -  | 2.21 |
| Cu–O                        | 3.58(0.02) | -  | 3.23 | -  | -  | -  | -  | -  | 3.87 |

| Angles (°)                  |            |    |    |    |    |    |    |    |
|-----------------------------|------------|---|---|---|---|---|---|---|
| His₁N⁶¹–Cu–His₂N⁶¹           | 106(1)     | 99(1) | 95 | 100 | 99 | 101 | 96 | 101 | 104 |
| His₁N⁶¹–Cu–CysS⁷             | 127.7(0.4) | 121.5(0.5) | 144 | 135 | 139 | 122 | 128 | 134 | 132 |
| His₁N⁶¹–Cu–MetS⁵             | 80(1)      | -  | 81 | 82 | 82 | 88 | 90 | -  | 84 |
| His² N⁶¹–Cu–CysS⁷            | 123(1)     | 126.3(0.8) | 104 | 120 | 103 | 114 | 107 | 118 | 111 |
| His₂N⁶¹–Cu–MetS⁵             | 98(3)      | -  | 116 | 97 | 117 | 116 | 130 | -  | 101 |
| MetS⁵–Cu–CysS⁷               | 108.5(0.8) | -  | 116 | 111 | 113 | 114 | 107 | -  | 117 |
| His₁N⁶¹–Cu–GlnOε¹            | -  | 103.4(0.9) | -  | -  | -  | -  | -  | -  | 94  |
| His₂N⁶¹–Cu–GlnOε¹            | -  | 101(1)    | -  | -  | -  | -  | -  | -  | 102 |
| GlnOε¹–Cu–CysS⁷              | -  | 95.4(0.7) | -  | -  | -  | -  | -  | -  | 101 |
| Dihedral angle (φ)²          | 81.5       | 89.3 | 64.1 | 79.4 | 64.7 | 74.3 | 65.8 | 83.5 | 79.3 |

²The structures of the T1Cu sites of Nir from Hyphomicrobium denitrificans (HdNir), Geobacillus. kaustophilus (GkNir), Alcaligenes. xylosoxidans (AxNir), A. faecalis (AfNir), Cucumis sativus stellacyanin (CsSte), Paracoccus versutus amicyanin (PvAmi) are taken from PDB entries 2DV6, 3WI9, 1OE1, 1SNR, 1JER and 1ID2, respectively. His₁ and His₂ are the first and second His ligands in the amino acid sequence. TsNirK ligand distances and bond angles are an average of all three monomers. ³Values in parentheses represents the standard deviation of measurements taken from the three monomers. ⁴The dihedral angle (φ) is the angle between the planes His₁N⁶¹–Cu–His₂N⁶¹ and L⁵axial–Cu–CysS⁷.
**Table S3**  Spectroscopic features of NirK and its comparison with other NirKs, stellacyanin and amicyanin.

| Protein* | TsNirK | HdNir | AxNir | A/Nir | CsSte | PvAmi |
|----------|--------|-------|-------|-------|-------|-------|
| Typeb    |        |       |       |       |       |       |
| Uv-Vis   |        |       |       |       |       |       |
| \(\lambda_1\) (nm) | 447    | 454   | 460   | 457   | 450   | 460   |
| \(\varepsilon_1\) (mM\(^{-1}\)cm\(^{-1}\)) | 1.86   | 2.9   | 1.6   | 6.98  | 1.1   | 0.43  |
| \(\lambda_2\) (nm) | 597    | 605   | 593   | 587   | 608   | 596   |
| \(\varepsilon_2\) (mM\(^{-1}\)cm\(^{-1}\)) | 9.09   | 6.30  | 6.30  | 5.42  | 4.08  | 3.90  |
| \(\varepsilon_1/\varepsilon_2\) | 0.21   | 0.46  | 0.25  | 1.29  | 0.27  | 0.11  |
| EPR (X-band) | 2.260; 5.9 | 2.21; 5.5\(^c\) | 2.208; 6.3 | 2.254; 7.4 | 2.290; 3.2 | 2.239; 5.6 |
| | 2.23; 6.0\(^d\) | | | | | |
| T2Cu \([g_e; A_g\) (mT)] | 2.296; 14.5 | 2.35; 13.5 | 2.298; 14.2 | 2.394; 13.0 | – | – |

*Copper nitrite reductases from *Hyphomicrobium denitrificans* (*HdNir*) (Yamaguchi *et al.*., 2004), *Alcaligenes xylosoxidans* (*AxNir*) (Abraham *et al.*, 1993), *A. faecalis* (*AfNir*) (Tocheva *et al.*, 2007), *Cucumis sativus* stellacyanin (*CsSte*) (DeBeer George *et al.*, 2003) and *Paracoccus versutus* amicyanin (*PvAmi*) (Buning *et al.*, 2000) are compared with *TsNirK*. b color of the protein: B, Blue; GB, greenish-blue; G, green. c,dThese values correspond to the values reported for C114A and C260A variants of *HdNir*, respectively (Yamaguchi *et al.*, 2004).
Figure S1  Molecular phylogenetic analysis by maximum likelihood method. The unrouted phylogenetic tree shows the distribution of classical NirKs and novel three-domain NirK. The proteins are named by UniProt code or PDB (crystal structure available) followed by a 5 letter code identifying the source. THESC: *Thermus scotoductus* SA-01, THEBO: *T. brockianus*, THEOS: *T. oshimai* JL-2, CREPO: *Crenothrix polyspora*, FRAsp: *Fraserbacteria* sp., CALSU: *Caldiaarchaeum subterraneum*, THIVE: *Thioalkalivibrio versutus*, CALTH: *Caldalkalibacillus thermarum*, ARMBA: *Aramatimonadetes bacterium* GSX, NITEU: *Nitrosomonas europaea*, MELTH: *Melghirimyces thermohalophilus*, PAEGL: *Paenibacillus glacialis*, GEOETH: *Geobacillus thermophilus*, GEOKA: *G. kaustophilus*, RHISU: *Rhizobium sullae*, SINME: Sinorhizobium meliloti 2011, RHIGA: *R. galagae*, ALCFA: *Alcaligenes faecalis*, ACHCY: *Achromobacter cycloclastes*, BRAJA: *Bradyrhizobium japonicum* USDA110, PSECL: *Pseudomonas chlororaphis*, ALCXY: *A. xylosoxidans*, RHOSP: *Rhodobacter sphaeroides*, HALME: *Haloraphis mediterranei*, HALMA: *Haloarcula marismortui*, POLNA: *Polaromonas naphtalenivorans*, NITMU: *Nitrospira multiformis* ATCC 25195, HYPDE: *Hyphomicrobium denitrificans* A3151, FUSOX: *Fusarium oxysporum*, CHRVI: *Chromobacterium violaceum*, RALSO: *Ralstonia solanacearum*, RALPI: *R. pickettii*, BURMA: *Burkholderia mallei* NTCT 10229, FLACO: *Flavobacterium columnare*, NEIGO: *Neisseria gonorrhoeae*, BDEBA: *Bdelvibrio bacteriovorus*, PSEHA: *Pseudoalteromonas haloplanctis*. 
Figure S2  Size exclusion chromatography of TsNirK. A prepacked SuperdexTM 200 10/300 G2 column (GE Healthcare) connected to an Akta prime (GE Healthcare) system was equilibrated with 200 mM NaCl 20 mM Tris–HCl buffer (pH 7.6). Isocratic elution at a flow rate of 0.5 mL min$^{-1}$ was performed with detection at 280 nm. The molecular weight markers used for calibration were ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa) and carbonic anhydrase (29 kDa) and ribonuclease A (13.7 kDa). SDS-PAGE gel is shown. Prestained mid-range protein marker (2-105 kDa, Genbiotech) was used as protein ladder.
**Figure S3** Domain I-domain III Interaction. Several amino acids of domain I (blue) and domain III (purple) are involved in the contact area. These amino acids interact through H-bonds or salt bridges (black dashed lines). Also, a few amino acids from domain III interacts with amino acids within domain II of neighbouring subunit (II.c, in grey). A number of water molecules (red spheres) take part in the interdomain interaction. The possible $\text{TICu}_C \rightarrow \text{TICu}_N$ electron transfer pathway is indicated in magenta involving His431, Glu385, His125 and a water molecule. His120 ($\text{N}^{\text{c2}}$)–Asn356 ($\text{O}^{\text{c1}}$), Ala124 ($\text{O}$)–Tyr314 (OH) and His125 ($\text{N}^{\text{c2}}$)–Glu385 ($\text{O}^{\text{c1}}$).
Figure S4  Details of the water channels in TsNirK. (a, b). Three channels allow the entrance of the substrate to the T2Cu active site pocket located at the region delimited by domains I and II. (c). These channels are located at the contact surface space between domains I and II of adjacent subunits (I.A-II.C; I.C-II.B; II.A-I.B). (d), Subunit A provides (purple): Leu77, Ser78, Thr86, Ser87, Gln91, Asn92, His114, Ala116 to Gly19, Ile122, Met123, Phe379, Leu382 and Arg386. Subunit C (yellow): Val188, His216, Val218, Asp261, Thr263, Val265, His267, Phe269, Leu277, Ile279 and Arg281. A network of H-bonded water molecules connects the mouth of the channel with the catalytic active site. The substrate sensing loop is located underneath the contact surface of subunit A and some of the residues interact with the water molecule network: Leu77, Ser78, Thr86, Ser87, Ala116, Gly119, Asp261 and Thr263. A hydrophobic lining spreads from the mouth of the channel trough Leu277, Ile279, Val188, Phe379, Leu381 and Leu382.
**Figure S5** Plot of initial velocities ($v$) of Nir activities for the $T_s$NirK vs nitrite concentrations. The solid line shown the fitted curved based on the standard Michaelis-Menten model. All the reactions were performed in mixtures containing 5.4 nM $T_s$NirK (trimer).
**Figure S6** Time course of the absorbance changes at wavelength 597 nm in Nir assay using SmPaz.

The reoxidation of SmPaz was followed at 597 nm. The mixture enzyme-SmPaz reduced with sodium dithionite was maintained under argon flux during 2 min then the reaction was started by addition of argon-flushed sodium nitrite solution. The green and blue lines are SmNir ($v_i = 0.55 \pm 0.01 \mu$M.s$^{-1}$) and TsNirK ($v_i = 0.075 \pm 0.009 \mu$M.s$^{-1}$) reactions, respectively. The black line corresponds to the reaction mixture with no enzyme as a reaction control. The measurements were performed in triplicates.
Figure S7  rR spectra calculated using QM/MM. The combination of Quantum Mechanics and Molecular Mechanics (QM/MM) calculations were used to compute the structure and the Raman spectra of the T1Cu_C and T1Cu_N sites in T3NirK. The main Cu–S (Cys) vibrational modes are indicated for each T1Cu centre.
**Figure S8** Relative peak intensities in the rR spectra of TsNirK and related copper proteins. Data taken from sources detailed in text. *Rhus vernicifera* stellacyanin (*Rv*Ste) (Nestor et al., 1984), *Cucumis sativus* stellacyanin (*Cs*Ste) (Nersissian et al., 1996), *Paracoccus denitrificans* amicyanin (*Pd*Ami) (Sharma et al., 1988), *P. versutus* amicyanin (*Pv*Ami) (Buning et al., 2000). Inserts in each panel shows the loop sequence that connects Cys with the axial ligand (Met or Gln) in T1Cu centres. Gray area represents the $\nu$(Cu–S) frequencies range for rhombic geometries and in yellow the axial geometry region (Andrew et al., 1994).