Supporting Information

Nitrite reductase activity in engineered azurin variants

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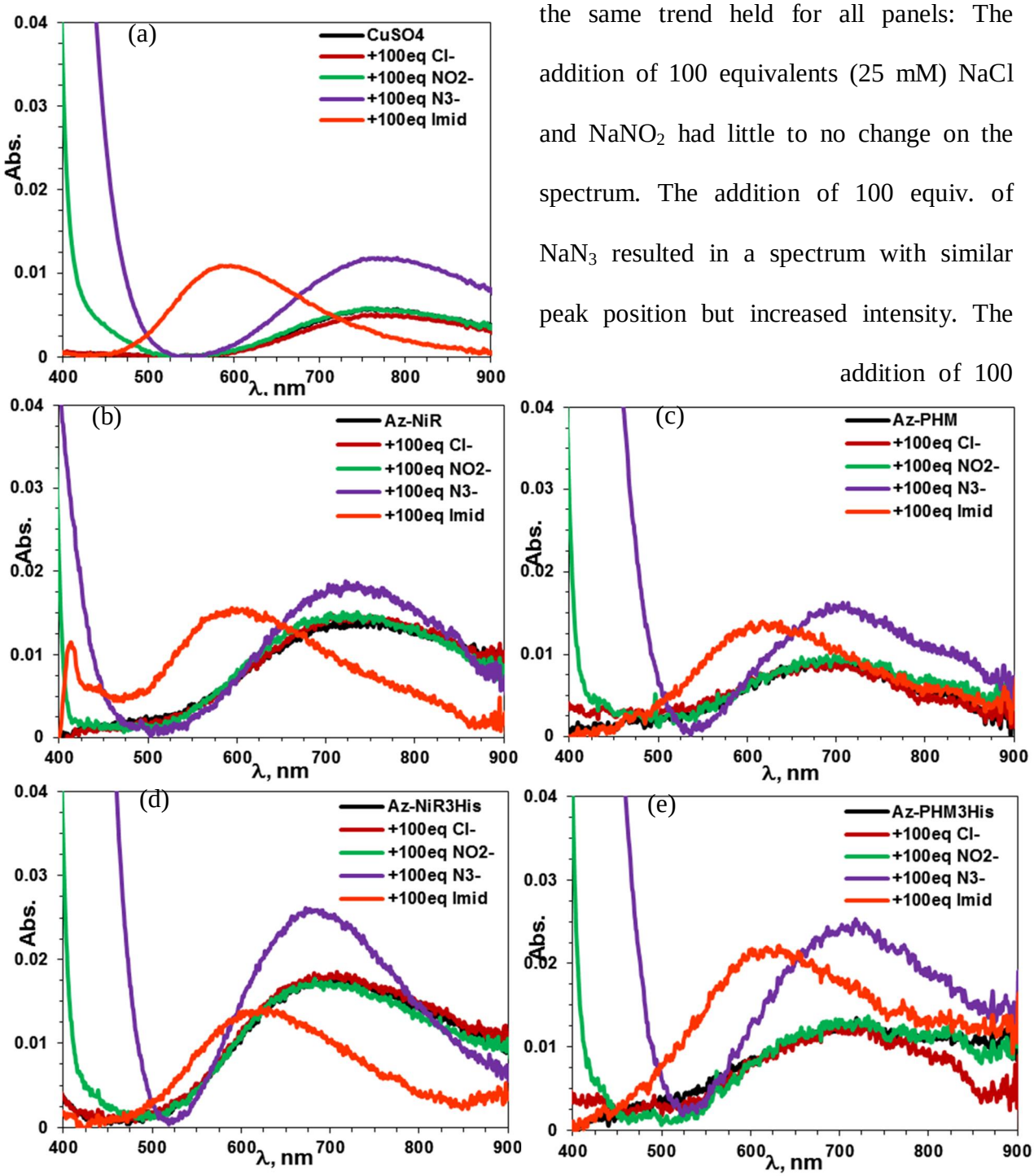
Table S1: Electrospray Ionization-MS data for the reported azurin variants. Samples were reconstituted with 1.5 equivalent of $CuSO_4$ and passed through a PD-10 desalting column prior to analysis. The samples were injected into the instrument in buffer, 50 mM ammonium acetate pH 5.1. Theoretical masses are for the +1 charged protein species with one Cu(II) ion bound in the T1 copper site. Experimental masses are for the major peak observed in the deconvoluted +1 ion mass spectrum, with an uncertainty of +/- 0.05%.

Variant	$[M+H^+]$: Theory (Expt. ±7 Da)
WT *P.a* Azurin	14006.4 (14006.3)
Az-NiR: Asn10His,Gln14Asp,Asn16His-azurin	14039.4 (14040.3)
Az-PHM: Gln8Met,Gln14His,Asn16His-azurin	14041.5 (14041.7)
Az-NiR3His: Asn10His,Gln14His,Asn16His-azurin	14061.5 (14062.6)
Az-PHM-3His: Gln8His,Gln14His,Asn16His-azurin	14047.4 (14048.6)

Figure S1. UV-visible absorption spectra of the four T1Hg(II)T2Cu(II)-azurin variants and free $CuSO_4$ upon the addition of small exogenous ligands NaCl, $NaNO_2$, NaN_3, and imidazole. Samples are 250 μM Cu(II) in 50 mM ammonium acetate pH 5.1. Panel a, b, c, d, and e show spectra for $CuSO_4$, Az-NiR, Az-PHM, Az-NiR3His, and Az-PHM3His, respectively. Generally, the same trend held for all panels: The addition of 100 equivalents (25 mM) NaCl and $NaNO_2$ had little to no change on the spectrum. The addition of 100 equiv. of NaN_3 resulted in a spectrum with similar peak position but increased intensity. The addition of 100



eq. of imidazole resulted in a blue-shifted peak (to about 600 nm) that resembled the spectrum of free $CuSO_4$ with imidazole in buffer. Gradual titrations (data not shown) of the protein with NaN_3 and imidazole from 1 equivalent to 100 equivalents resulted in a gradual shift of the absorption spectrum form that of the protein alone, to that shown in the figures for that of the 100 equivalent spectrum.

Figure S2. X-band EPR spectra of the four T1Cu(II)T2Cu(II)-azurin variants upon the addition of small exogenous ligands $NaNO_2$, NaN_3, and imidazole. Proteins are ~500 μM in 50 mM ammonium acetate pH 5.1 buffer. Panels a, b, c, and d include spectra for Az-NiR3His, Az-PHM3His, Az-NiR, and Az-PHM, respectively.

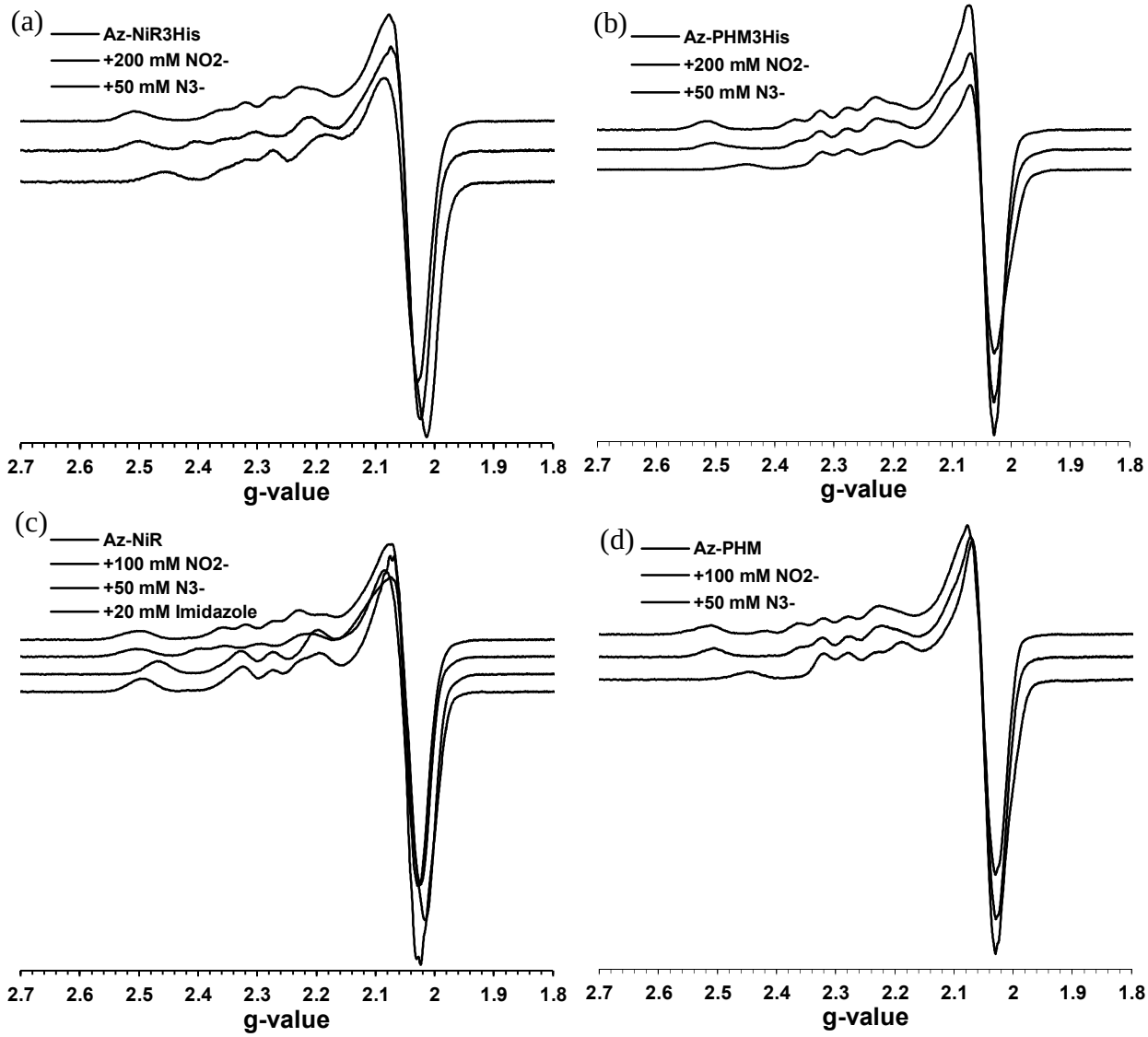


Figure S3. UV-visible absorption spectra of a cuvette baseline with an $[Fe(EDTA)]^{2-}$ solution. Solid Curve: The UV-visible absorption spectrum of the $[Fe(EDTA)NO]^{2-}$ complex generated from bubbling 1 mL of head space gas from a reaction with 260 µM Az-NiR3His, 100 mM nitrite, and 30 mM ascorbate into an $Fe[EDTA]^{2-}$ solution.[1,2] The spectrum was consistent with that observed by others.[3-5] Dashed Curve: A control spectrum after 5 mL of the gas from the anaerobic chamber was bubbled through the $[Fe(EDTA)]^{2-}$ solution.

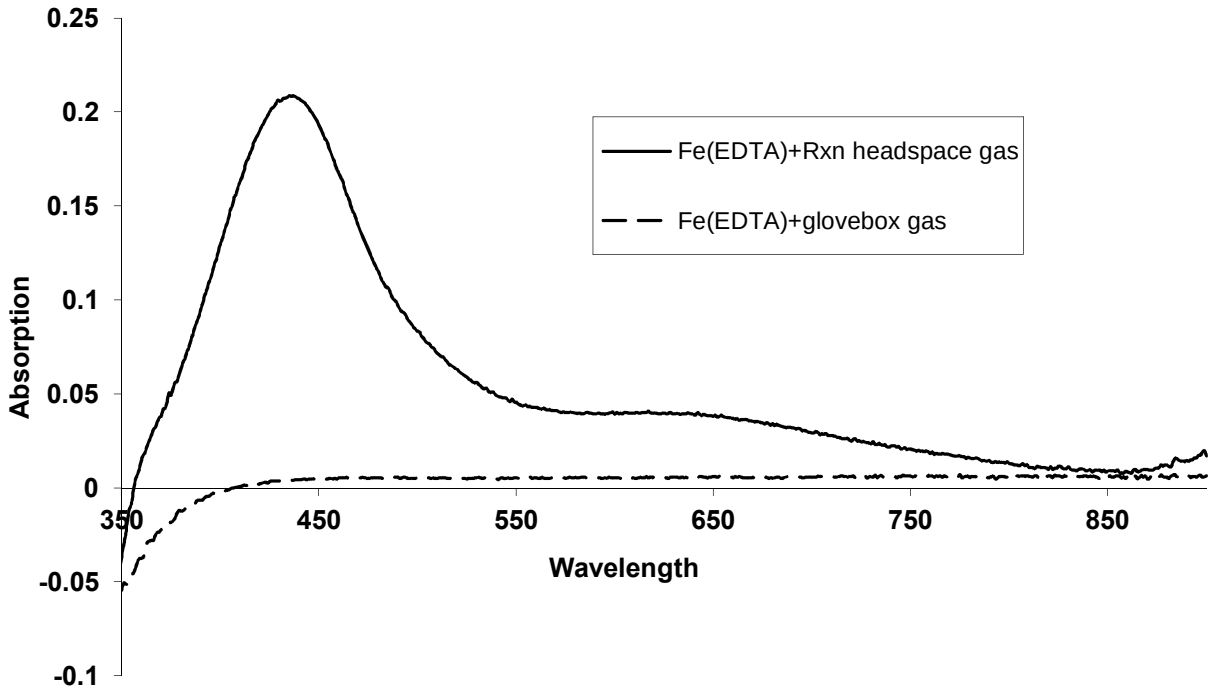


Figure S4. Michaelis-Menten plot for nitrite reduction by (left) 86 μM WT azurin and (right) no catalyst in 20 mM phosphate buffer at pH 6.35 with 33 mM ascorbate. WT azurin was reconstituted with Cu(II) ions (5 eq) and passed through a desalting column in an identical manner to the variants. At each nitrite concentration, the consumption of nitrite by ascorbate over 2 hours was essentially negligible. With no catalyst, the loss of the highest nitrite concentration (100 mM) by ascorbate over 2 hours was less than 0.05%.

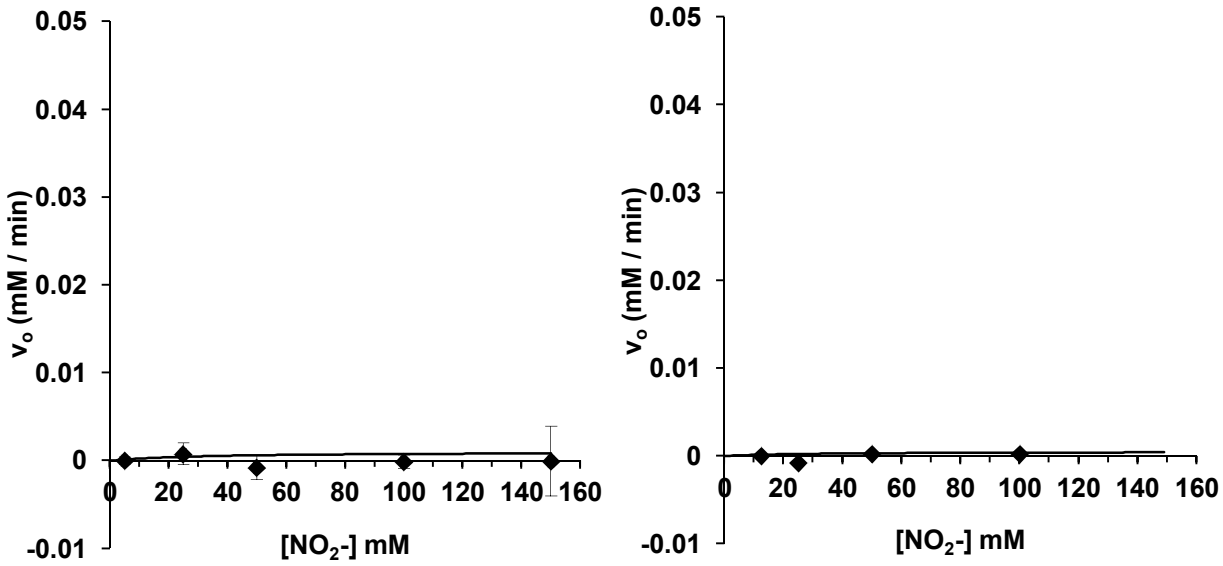
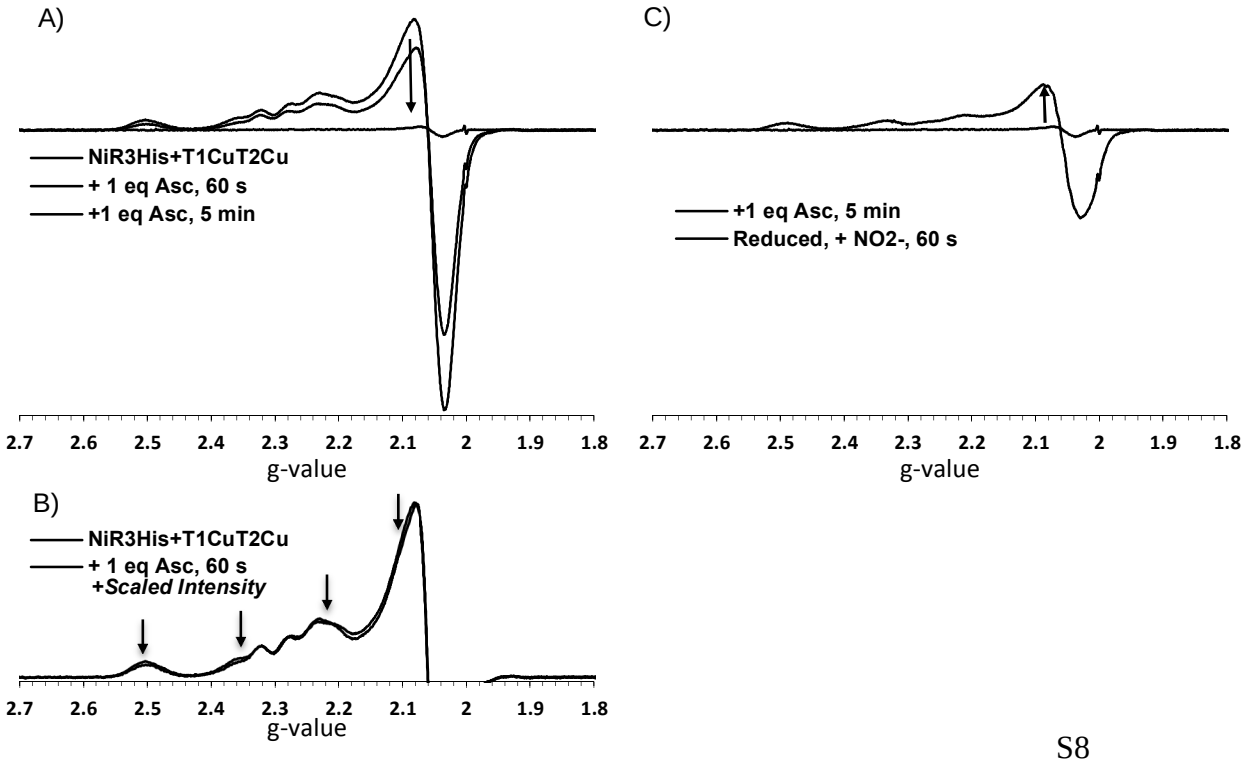


Figure S5. EPR study of the reduction of Az-NiR3His with ascorbate, followed by reoxidation with NO_2-. Resting Az-NiR3His with T1CuT2Cu (Panel A, top spectrum) showing the overlapping T1 and T2 copper signals. One equiv. of ascorbate was added and after mixing for ~60s, the T1 Cu blue color reduced ~15% (A_{625}~4.3 to ~3.6). EPR showed decreased T1 and T2 copper signals (A, middle spectrum). However, the T2 Cu signal (with broader hyperfine splitting) was more reduced than the T1 Cu signal (Panel B, lower spectrum with scaled intensity to show decreased T2 Cu relative to T1 Cu signal). After about 5 min, the sample was ~95% reduced (A_{625}~0.15) (panel A, lower spectrum and panel C, lower spectrum).

Sodium nitrite (10 mM) was then added to the fully reduced sample. After 60s of mixing, the blue T1 Cu began its recovery. The reaction was frozen at ~10% oxidized T1Cu (A_{625} ~0.15 to 0.5). The EPR spectrum (Panel C, top spectrum) indicated the presence of significantly more reoxidized T2 Cu than T1 Cu.

Taken together, these data indicate that the T2 copper is reduced and reoxidized ahead of the T1 copper center, particularly in the reoxidation reaction with nitrite.



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