SUPPLEMENTAL DATA

THE MOLECULAR MECHANISM OF 14-3-3-MEDIATED INHIBITION OF PLANT NITRATE REDUCTASE

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EXPERIMENTAL PROCEDURES

Determination of reduction potentials- Reduction potentials for the heme site in the presence and absence of phosphorylation/14-3-3ω were determined using the method described by Massey (1) at approximately 22°C in assay buffer. Briefly, the fragment to be investigated at a concentration of 5-10 μM and in the presence of an equal concentration of an appropriate dye of known reduction potential (indigo carmine, -116 mV at 20-25^{o}C (2)), 200 μM xanthine, and 2 μM methyl viologen as mediator were made anaerobic in a tonometer as described before, with a stock solution of xanthine oxidase sufficient to give a final concentration of 10-20 nM in the sidearm. The spectrum of the oxidized mixture was recorded, after which the xanthine oxidase solution was tipped in and spectra recorded using a Hewlett-Packard diode array spectrophotometer in the kinetics mode for 2-4 hours monitoring at 425 nm (for heme) and 630 nm (for the dye) as the fragment and dye were slowly reduced by the xanthine/xanthine oxidase mixture. A fully reduced spectrum of the mixture was obtained when the reaction was deemed nearly completed by introduction of a small amount of a concentrated solution of sodium dithionite. The reduction potential for the heme was determined from the y-intercept of plots of the log ox/red of normalized dye vs. that of heme according to the following form of the Nernst equation, $E_h = E_m + (2.303\ RT/nF)*\log([\text{oxidized form}] / [\text{reduced form}])$, where E_h is the calculated redox potential, E_m is the reduction potential of the known dye, and n is the number of electrons involved in the electron transfer (Figure S1).

Immunoblots- Recombinant NR and phosphorylated NR variants (15 μmol each) were separated via 12% or 8% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinyl difluoride membranes. The membranes were incubated in blocking buffer (5% (w/v) milk in 50 mM Tris, 150 mM NaCl, 0.05 % Tween 20) at room temperature for 1 h. For detection of His_6-tagged proteins, Ni-nitrilo-triacetic acid alkaline phosphatase conjugate (Qiagen) was used in a dilution of 1:1000 in 50 mM Tris, 150 mM NaCl, 0.05 % Tween 20 and incubated for 1 h at room temperature. Afterward, the membranes were developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium chloride (AppliChem) as substrates.

Detection of phosphorylated protein was performed using a polyclonal antibody from rabbit directed against a peptide (residues 529-541) including phospho-Ser534 (Eurogentec) in a dilution of 1:25000 and incubated for 1 h at room temperature. As secondary antibody anti-rabbit IgG alkaline phosphatase conjugate (Sigma) (dilution 1:20000) was used with similar incubation conditions before the membranes were developed (Figure S2).

SUPPLEMENTAL TABLE

Table S1: Steady-state kinetic parameters.

sample	$K_M^{nitrate}$ [µM]	K_M^{NADH} [µM]	k_{cat} [s^{-1}]
holo-NR, untreated[1]	160 ± 13	-	34 ± 1
holo-NR in 35% glycerol[1]	77 ± 17	-	12 ± 1
Mo-heme, untreated[1]	164 ± 31	-	35 ± 2
Mo-heme in 35% glycerol[1]	65 ± 22	-	12 ± 1
Heme-FAD, untreated[2]		20 ± 5	58 ± 6
Heme-FAD, in 35 % glycerol[2]		14 ± 6	57 ± 8

[1] measured with methyl viologen:nitrate assay; [2] measured with NADH:cytochrome *c* assay

SUPPLEMENTAL FIGURES

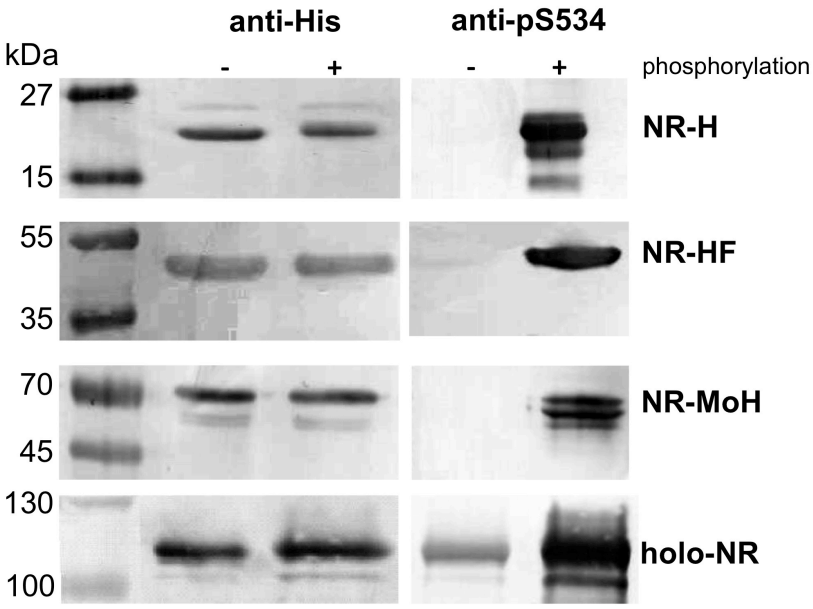

Fig. S1: Immunoblots of NR variants using anti-His or anti-pSer534 antibodies. All fragments used in this work were applied directly (= untreated) or after phosphorylation (indicated with - or +) to SDS-PAGE and separated. One sample was then subjected to immuno-staining using the anti-His_6 conjugate or an antibody specifically against the phosphorylated Ser534 region demonstrating that all fragments were phosphorylated. Only holo-NR displays a phospho-signal before phosphorylation treatment that is due to intrinsic phosphorylation during expression in *Pichia pastoris*. Protein sizes of the used standard proteins are labeled on the left.

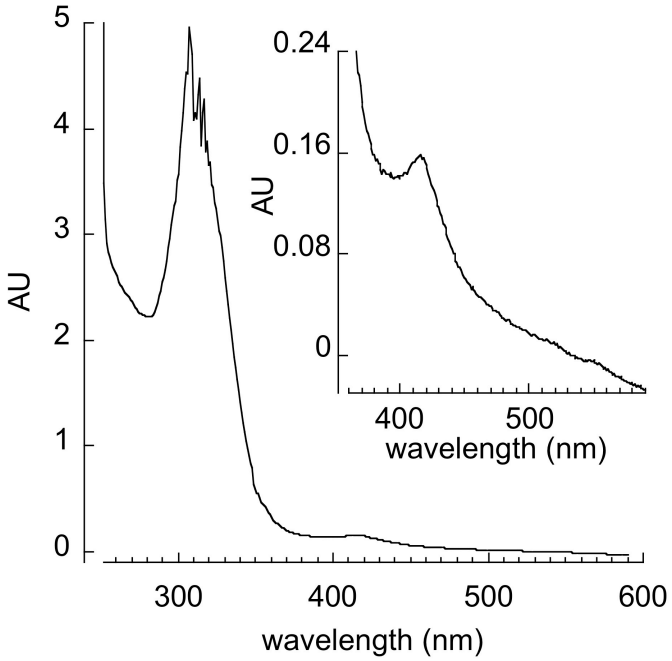

Fig. S2: uv/visible spectrum of 41 μM purified Mo-heme His600Ala variant. The inset shows the presence of trace amounts of heme.

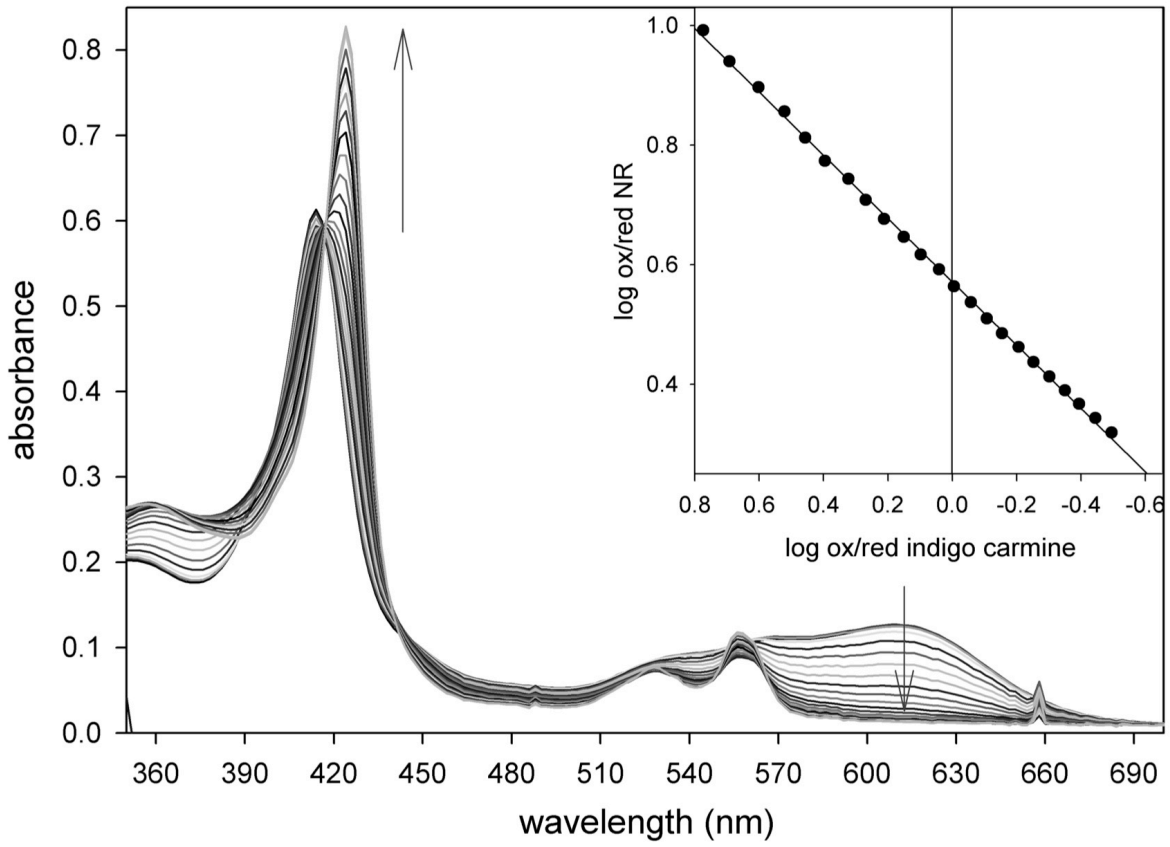

Fig. S3: Representative plot of redox potential determination. Slow reduction of 5 µM heme/5 µM of indigo carmine by the xanthine/xanthine oxidase mixture. The reduction potential for the heme was determined from the y-intercept of the plot of the log ox/red of the dye vs. that of the heme (inset).

Supporting References

1. Massey, V. (1991) *Flavins and Flavoproteins 1990*, 59-66
2. Guengerich, F. P., Ballou, D. P., and Coon, M. J. (1975) *J Biol Chem* **250**, 7405-7414