Supporting Information for the World Wide Web Edition of Mechanism of $p21^{Ras}$ S-nitrosylation and Kinetics of Nitric Oxide-mediated Guanine Nucleotide Exchange on $p21^{Ras}$ by Jongyun Heo and Sharon L. Campbell[*,‡]

Materials and Methods

Quantification of NO. The Hb-coupled assay was modified to quantify NO under anaerobic conditions and low redox potential. The binding affinity of the reduced Hb (containing Fe^{2+}-heme moiety) to NO is at least 100-fold stronger compared to that of the oxidized Hb (containing Fe^{3+}-heme moiety) (*1*). The midpoint potential ($E_m°'$) for Fe^{2+}-heme/Fe^{3+}-heme couple is +102 mV SHE (*2*). Therefore, the redox potential of the Hb stock solution (3 mL) was adjusted to –500 mV (versus SHE) by adding sodium dithionite (1 mM), to produce reduced Hb, with the final Hb concentration in the solution adjusted to 0.2 mg of Hb/mL. A NO-saturated standard solution was prepared in a sealed O_2-free vial (50 mL) by purging NO gas for 20 min. The saturated NO concentration in water is known to be 2 mM at 25ºC and P_{NO} = 1 atm (*3*). A standard curve for NO quantification was generated by adding a known amount of NO-saturated solution (typically 1-5 μL) into the Hb-containing assay cuvette. The Soret absorption peaks corresponding to reduced Hb at 433 and 419 nm were proportionally changed upon the binding of NO to the reduced Hb, and monitored using a Beckman DU650 spectrophotometer (Supporting Information, Fig. 1). The NO-dependent absorption change of reduced Hb at 419 nm and 433 nm is directly proportional to NO concentrations in a range of nM to 100 μM with a linear correlation factor of $R^2 > 0.9950$ (Supporting Information, Fig. 1 inset). To quantify the NO content in the assay, an aliquot of the NO-containing assay mixture (1-50 μL) was transferred to the Hb-assay

solution, and the corresponding Soret band shifts were monitored. Comparison of the sample Soret band shifts to a NO standard curve provided the quantity of dissolved NO in the assay solution. For experiments using a mixture of NO and O_2, the dissolved NO content in assay solutions was measured prior to add O_2.

Quantification of S-nitrosylated Compounds. A spectroscopic method monitoring absorption intensity at 336 nm to detect S-nitrosothiol species is a well-established method to quantify LMW RSNO (*4*). However, quantifying the fraction of protein S-nitrosylation by monitoring absorption at 336 nm could generate significant errors because proteins rich with Phe, Tyr, and Trp, or containing transition metals as prosthetic groups, typically have strong absorption near 336 nm. Thus, we applied a practical method for the quantification of PSNO by monitoring the absorption peak at 542 nm (Supporting Information, Fig. 2). The absorption spectrum at near 540 nm is most likely due to the $n \rightarrow \pi^*$ transition of the SNO moiety (*5*). The intensity at 542 nm is linearly proportional to the content of SNO with an extinction coefficient (ε) of 0.019 $cm^{-1}mM^{-1}$, indicating that analysis of the peak intensity at 542 nm can used to quantify the S-nitrosylated compounds (Supporting Information, Fig. 2A inset). A similar extinction coefficient of 0.020 $cm^{-1}mM^{-1}$ at 542 nm was determined for S-nitrosylated Ras, which corresponds to S-nitrosylation of a single Ras PSH residue (Supporting Information, Fig. 2B inset).

Kinetic expressions for solvent accessible exposed thiol-specific NO-mediated S-nitrosylation can be described below (*6*):

$$\mathrm{PSH + NO} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{PSH{\cdot}NO} \xrightarrow{k_2} \mathrm{PSNO + H^+ \ (or\ H^{\bullet})} \quad (1)$$

$${}^{specific}K_{\mathrm{NO}} = \frac{[PSH][NO]}{[PSH \cdot NO][H^+]}, \text{ and thus } k_{observed} = \frac{k_3 \cdot [NO]}{K_{NO} + [NO]} \quad (2)$$

where PSH·NO is the noncovalent complex, and PSNO is the covalently bonded S-nitrosylated end product of the reaction (S-nitrosylated PSH). The reaction process, $k_{2,}$ may require an electron acceptor as shown in Scheme 1B as well as 1D. k_{observed} can be expressed as a quantity of S-nitrosylated product monitored at 542 nm (${}^{specific}Abs_{542}$):

$${}^{specific}Abs_{542} = \frac{k_2 \cdot [NO]}{K_{NO} + [NO]} \quad (3)$$

A minor component of Abs_{542} resulting from nonspecific modification of PSHs (i.e., a minor modification at additional PSH sites) with NO will be treated as:

$${}^{nonspecific}Abs_{542} \cong {}^{nonspecific}K_{NO} \cdot [NO] \quad (4)$$

The total quantity of S-nitrosylated product (L_{T}) can be expressed as follows by combining the Equation (3) and (4):

$$L_T = \frac{k_2 \cdot [NO]}{K_{NO} + [NO]} + {}^{nonspecific}K_{NO} \cdot [NO] \quad (5)$$

Supporting Data

Supporting Scheme and Figures

Scheme 1. The interactions between the NKCD and the SA motifs of Ras with GDP. Hydrogen bond interactions between the Ras NKCD motif and GDP are presented as dotted lines, based on a high-resolution crystal structure (PDB 1QRA).

Figure 1. Quantification of dissolved NO. An aliquot (2 μL) of NO-saturated standard solution (2 mM [NO]) was injected into a sealed O_2-free assay cuvette (3 mL size) that contained a reduced hemoglobin (Hb)-assay solution. The Soret spectrum of the reduced Hb in a range of 400 to 480 nm was monitored before and after addition of NO gas. Four different volumes of NO (1-10 μL) gas were repeatedly injected into the same sample vial, and the corresponding Soret spectra were recorded. The Soret responses to NO adduction are plotted against NO concentrations (inset).

Figure 2. Visible spectra of S-nitroso compounds. GSNO (1 mM) and the Ras protein (20 μM) were prepared in an O_2-free sealed assay cuvette (1 mL size). GSNO (A) and Ras-S-NO (B) were scanned in the range of 400 - 800 nm. As indicated by the arrow, a peak at 542 nm is detected. The peak intensity is linearly proportional to the concentration of GSNO with an extinction coefficient of 0.019 $cm^{-1}mM^{-1}$ (inset).

Figure 3. Quantification and comparison of NO/O_2-mediated wt and D119N Ras S-nitrosylation. Expression and purification protocols for D119N Ras were identical to those employed for wt and C118S Ras, as described in Materials and Methods. Experimental conditions and procedures for quantifying NO/O_2-mediated S-nitrosylation of D119N Ras are also described in Fig. 1 of the main article. For comparison, NO/O_2-

mediated S-nitrosylation of both wt Ras and D119N Ras are plotted. The plot was fit to Eqn. 5. The values shown in this Figure represent mean values of measurements conducted in triplicate with their standard errors.

References for Supporting Information

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Scheme and Figures for Supporting Information

Scheme 1.

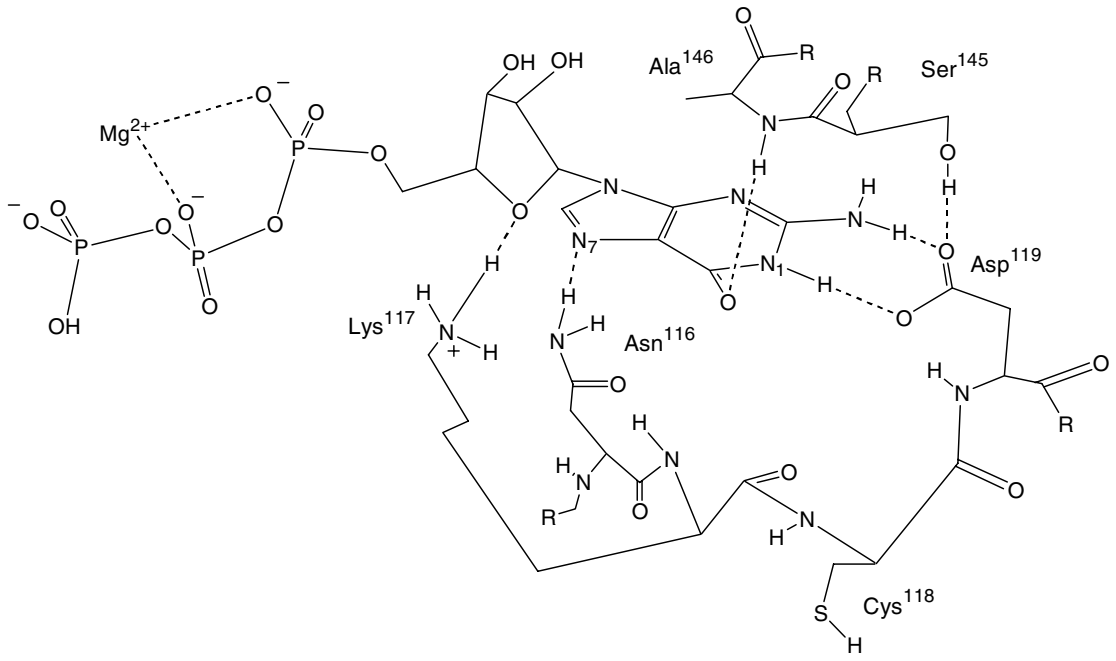


Figure 1.

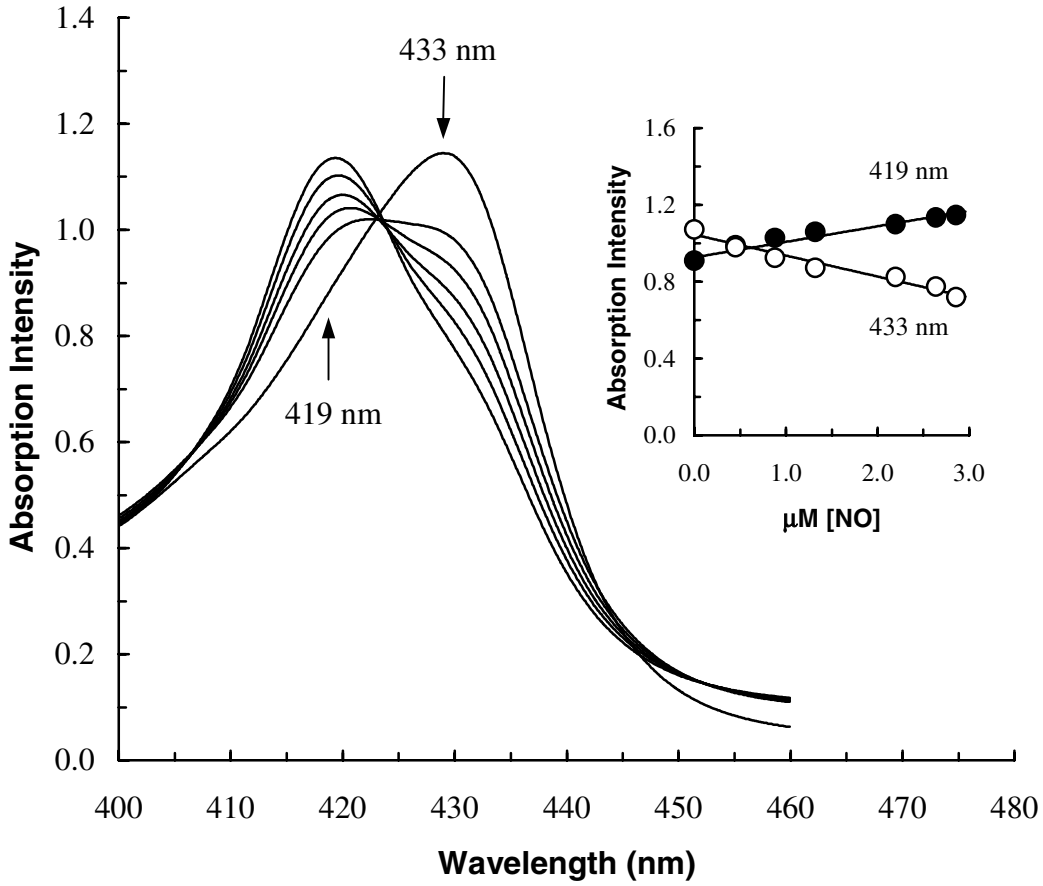

433 nm
419 nm
Absorption Intensity
Wavelength (nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
400
410
420
430
440
450
460
470
480
419 nm
433 nm
Absorption Intensity
μM [NO]
1.6
1.2
0.8
0.4
0.0
0.0
1.0
2.0
3.0

Figure 2.

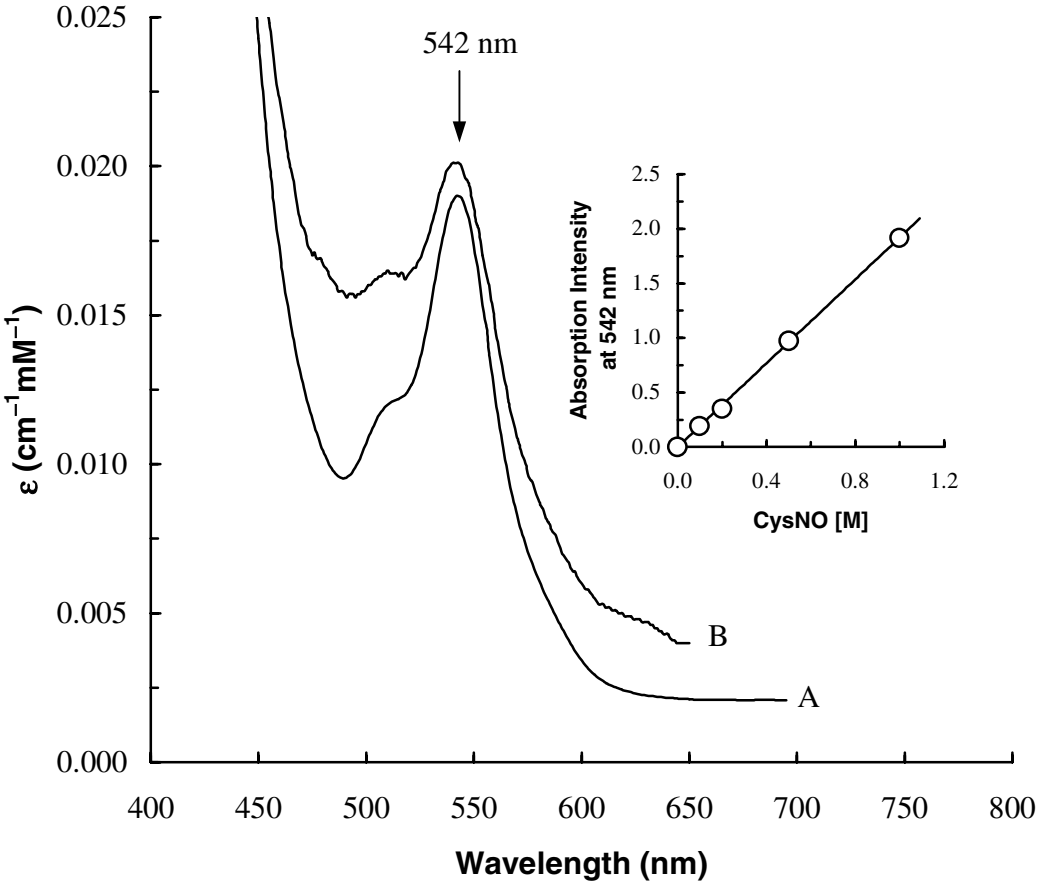
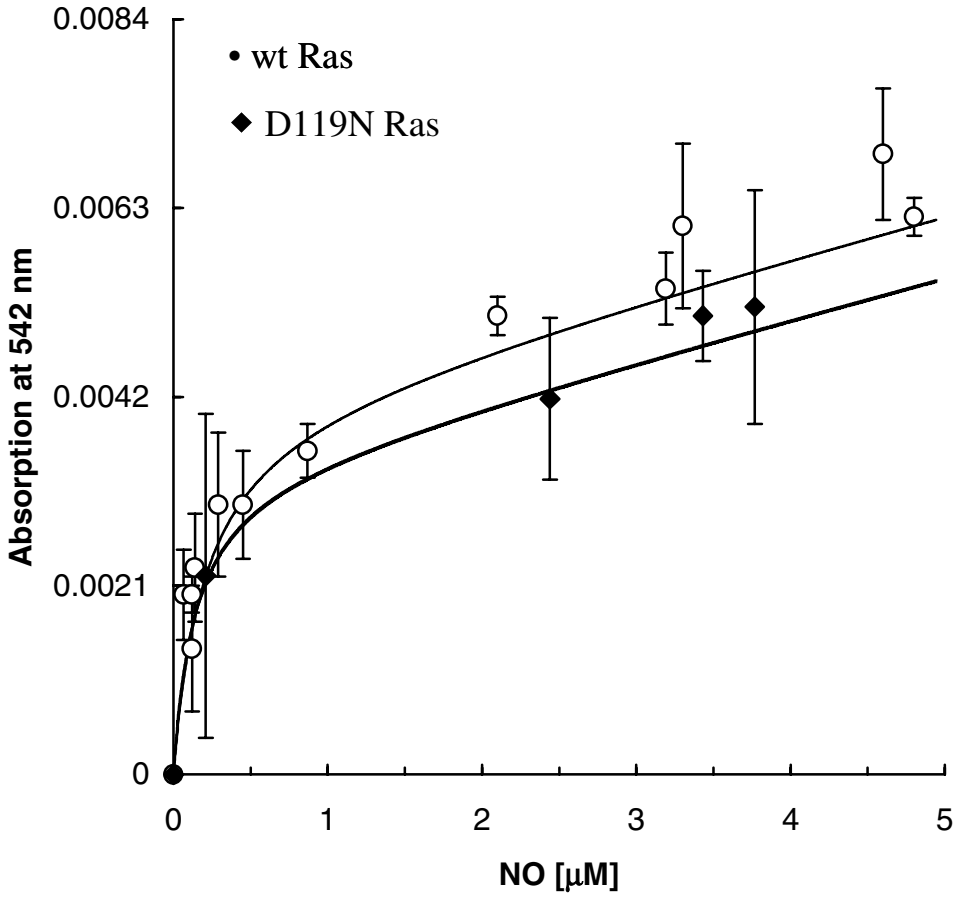

542 nm
B
A
ε (cm⁻¹mM⁻¹)
Wavelength (nm)
Absorption Intensity at 542 nm
CysNO [M]

Figure 3.


0.0084
0.0063
0.0042
0.0021
0
Absorption at 542 nm
• wt Ras
◆ D119N Ras
0
1
2
3
4
5
NO [μM]