Supporting Information

The Role of Arg127 at the Proximal NO Binding Site in Determining the Electronic Structure and Function of 5-Coordinate NO Heme in Cytochrome c′ of *Rhodobacter sphaeroides*

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Cytochrome c′ Mutagenesis and Expression

Materials for Mutagenesis

Bacterial strains. A knock-out *R. sphaeroides* 2.4.3 strain, in which the gene encoding Cyt c′, *cycP*, was insertionally inactivated was used for expression of Cyt c′ via the *cycP* and *rrnb* system described earlier. *E. coli* (S17-1) was used as a donor in conjugations to mobilize the pRK145 plasmid containing *cycP* and *rrnb* into the Cyt c′ knock-out of *R. sphaeroides* 2.4.3. *E. coli* (S17-1) and the *R. sphaeroides* 2.4.3(*1, 2*). *E. coli* DH5α was used to maintain a smaller pUC19 plasmid containing wild type *cycP* and *rrnb*. *E. coli* (XL-10 Gold) from Stratagene were used for transformations involving pUC clones.

Plasmids. pUC19, containing *cycP*, the *rrnb* promoter, and ampicillin resistance, was used as a template of one-site Quick-Change® (Stratagene) mutagenesis of *cycP*. pUC19 was sufficiently small (2.6 kbp) to be amenable to the Quick-Change® method. The pRK415 plasmid, containing tetracycline resistance, was used as an expression vector for *cycP* and its *rrnb* promoter within the *cycP* mutant of *R. sphaeroides* 2.4.3 strain. pRK415, due to its large 10.5 Kbp size, was not used for Quick-Change® mutagenesis; rather, *cycP* and the *rrnb* promoter were transferred to pRK415 by a restriction/religation process. pRK415 is a broad host range vector that is stable in both *E. coli* (S17-1) and *R. sphaeroides*.

Primers and Polymerases. Phusion DNA polymerase from New England Biolabs was used for PCR in DNA sequencing and PfuTurbo DNA polymerase from Stratagene was used for PCR in mutagenesis. EcoRI and HindIII restriction enzymes and T4 ligase were from Fermentas, Inc. All primers were obtained from Invitrogen. QIAGEN kits were used for gel and plasmid extraction according to the manufacture's specifications. PCR was performed with a

Gene Amp System 2400 Thermocycler (Perkin Elmer). DNA sequencing was done using a ABI 3730 DNA Sequencer and analyzed by 3730/3730xl Data Collection Software.

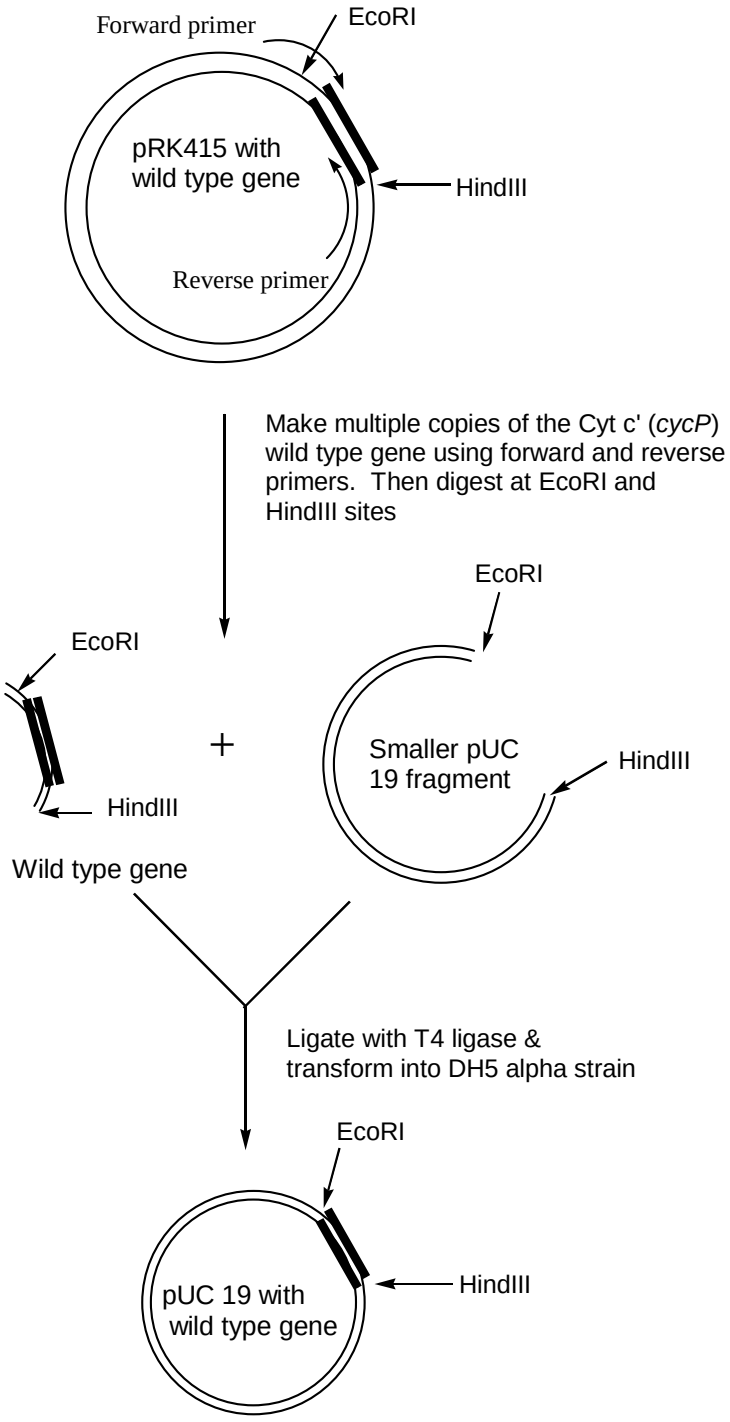
Mutagenesis

Site directed mutagenesis at position 127 of *cycP* and reincorporation of *cycP* into *R. sphaeroides* were achieved by steps described in **Scheme 1, 2, 3**. The detailed conditions for various procedures including concentrations, solution volumes, and incubation temperatures are provided in the B. Lee Ph.D. Thesis(*3*). In order to obtain sufficient quantities of the relevant genes (*cycP* and *rrnB*), the coding region of *cycP* and *rrnB* inserted in pRK415 was amplified following HindIII and EcoRI digestion, as indicated in **Scheme 1**. The amplified wild type gene was next digested using EcoRI and HindIII to produce sticky ends for ligation into pUC19 digested with the same enzymes. This subsequent ligation resulted in pUC19 containing wild type *cycP* and *rrnB,* as indicated in the bottom of **Scheme 1**.

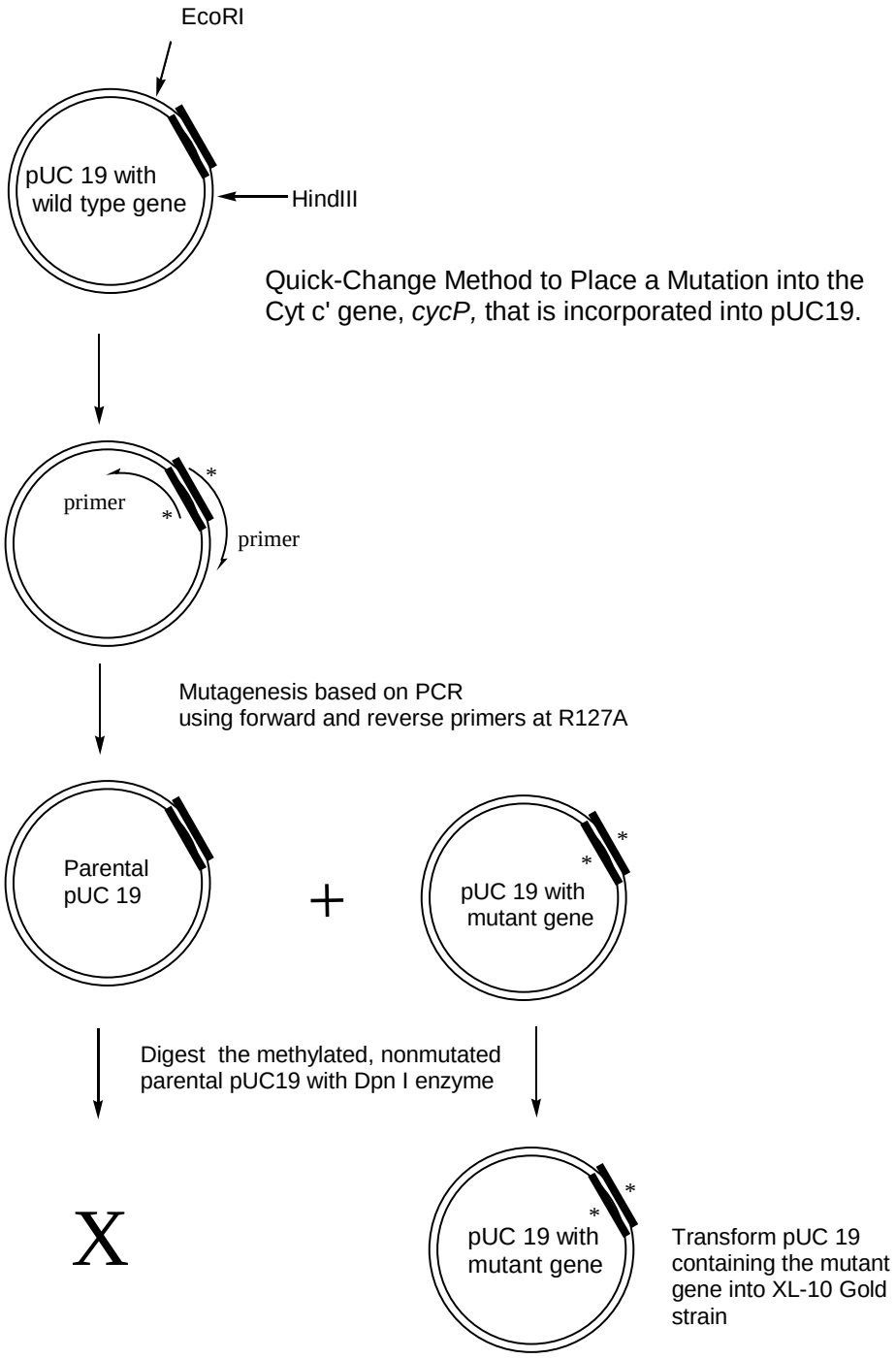
pUC19 was transformed into DH5α competent cells for amplification of the pUC19 plasmid as indicated in **Scheme 1**. Following selection for ampicillin resistance, DNA sequencing was performed to verify that the pUC19 contained the *cycP* and *rrnB* insert. At this point mutagenesis was performed on *cycP* in pUC19 using the Quick-Change® site-directed mutagenesis kit from Stratagene. The forward primer sequence for R127A mutation was 5′ C TGC CAC GAC GAC TTC **GCC** GCG AAG AAC TGA G 3′, and the reverse primer sequence for the R127A mutation was 5′ C TCA GTT CTT CGC **GGC** GAA GTC GTC GTG GCA G 3′. Subsequent PCR amplified the entire pUC19 plasmid, as shown in **Scheme 2**. As shown in **Scheme 2**, wild type plasmids were eliminated with *Dpn I*, which recognizes and cuts DNA isolated from the remaining pUC19 plasmids with the R127A mutation were transformed into *E. coli* (XL-10 Gold) provided by Stratagene. DNA sequencing of plasmids verified the mutation

from R127 to A127 in *cycP*. To move the mutated cycP-rrnB region into pRK the R127A fragment in pUC and pRK were separately digested using EcoRI and HindIII. The 898 bp *cycP/rrnB* region of pUC19 and the pRK415 plasmid flanks were purified by agarose gel electrophoresis and purified by the QIAquick gel extraction kits.

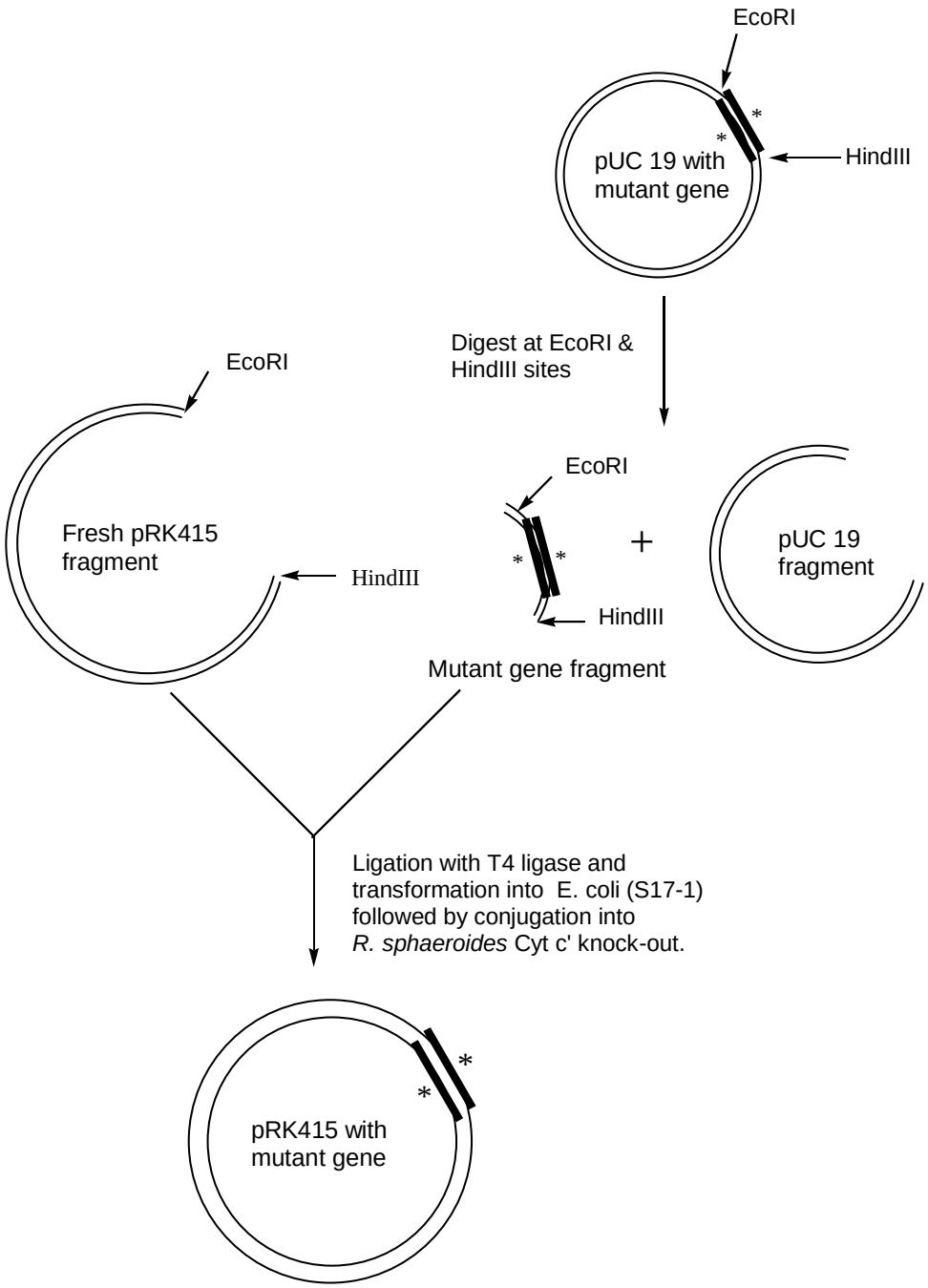
The R127A mutant gene contained in the 898 bp *cycP/rrnB* region was ligated into the pRK415 expression plasmid at the EcoRI and HindIII restriction sites. The pRK415 plasmids containing the mutant R127A gene were transformed into *E. coli.* (S17-1) as shown in **Scheme 3.** The pRK415 plasmids were sequenced to verify the R127A mutation within *cycP* was contained in pRK415. Next the *E. coli* (S17-1) containing pRK415 with the mutant gene was conjugated with the *R. sphaeroides* 2.4.3cycP mutant. The presence of pRK415 within mutant *R sphaeroides* 2.4.3, which was verified by tetracycline resistance and sequencing of the *cycP* gene from the pRK415, enabled expression of the mutant Cyt c′ protein.



Scheme 1. pUC19 Template Construction for CycP Mutagenesis



Scheme 2. Site-directed mutagenesis of *cycP* in the modified pUC19.



Scheme 3. pRK415 *cpcC* mutant plasmid construction for conjugation into a *R. sphaeroides* 2.4.3 Cyt c' knock out.

Expression and Purification

The *R. sphaeroides* 2.4.3 strains containing pRK415 were incubated in 4 L of Sistrom's medium (*4*), containing 1 μg/mL of tetracycline, 50 μg/mL of streptomycin and spectinomycin, for 3 days at 30 °C. Cells were harvested by centrifugation for 30 min at 10,000 g at 4 °C, washed in 20 mM sodium phosphate, pH 7.2 1 mM EDTA. Resuspended cells in the same buffer were disrupted by a French Pressure cell at 1400 psi. Cell extracts were obtained by an initial centrifugation for 10 min at 10,000 g and a second centrifugation for 2 hours at 256, 000 g. Ammonium sulfate was added to supernatants at 55 % percentage saturation at 4 °C and the supernatants were centrifuged for 10 min at 10,000 g at 4 °C. Ammonium sulfate was added to the supernatant to 85 % percentage saturation and the resultant precipitated proteins were pelleted by centrifugation for 10 min at 14,000 g at 4 °C. The pellet was dialyzed versus 20 mM sodium phosphate, pH 7.0 for 12 hours. A DEAE Fast Flow 7 ml column was used to remove unwanted paramagnetic proteins. Both the Cyt c′ wild type and R127A mutant were bound to the column at low ionic strength using 0.03 M pH 7.2 Tris buffer and eluted at 0.03 M pH 7.2 Tris, 0.07 M NaCl buffer (*1*).

SDS PAGE electrophoresis was used to monitor the purification of Cyt c′, and the molecular weights of both wild type and mutant (R127A) were estimated at14 kDa (**Figure 1S)**. The heme contents of both wild type and mutant proteins were determined by the pyridine hemochrome assay (25% pyridine, 80mM NaOH), in which the amounts of the protein-bound heme was obtained using E_{mM} = 29.9 at 550nm (*5*).

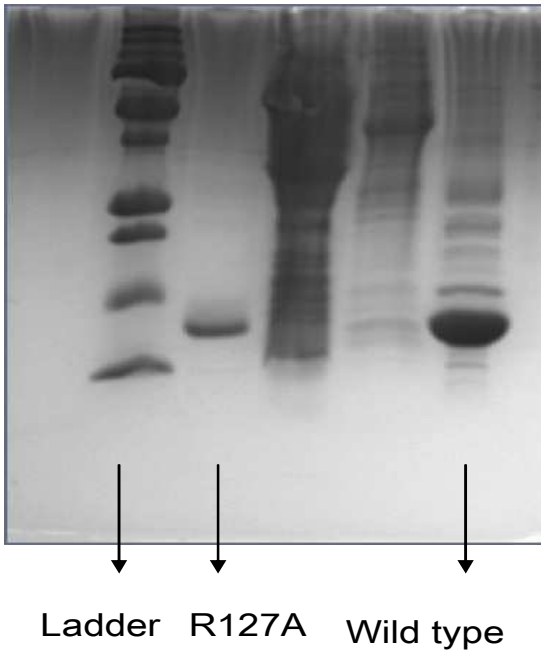


Figure 1S. SDS PAGE gel electrophoresis of wild type and R127A mutants from DEAE purification. The last two bands of the ladder on the left are 10 and 20 kDa markers. Lanes three and four are from an *R. sphaeroides* that did not express Cyt c'.

Optical Spectra - Soret Band Comparison of NO-Bound Wild Type and R127A Mutants

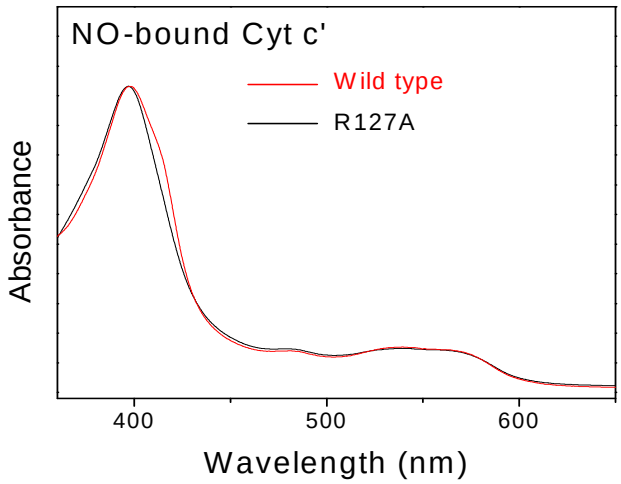


Figure 2S. UV-Vis absorption spectra NO-bound Cyt c' from wild type (Red) and R127A (black), where peaks at 397 nm are superimposed. The wild type protein shows a slight shoulder at ~414 nm, possibly due to a small amount of 6-coordiniate NO-heme-his.

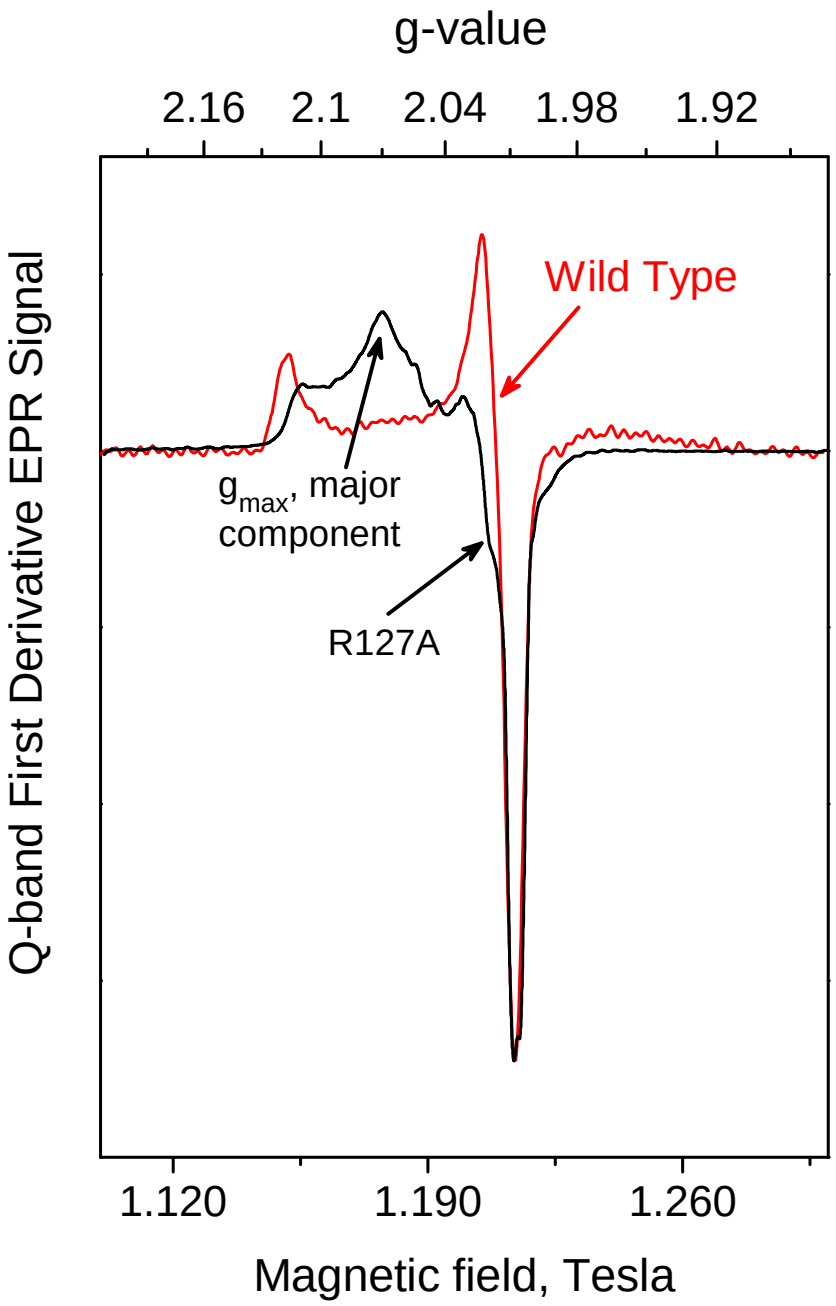


Figure 3S. These figures compare the first derivative of the Q-band rapid passage dispersion spectrum from NO-ligated wild type and R127A mutants. The EPR spectrum was obtained at T = 2 K, 2 G field modulation, 200 s of signal averaging with a 0.200 T sweep, 0.24 μW microwave power, EPR frequency ν_{EPR} = 34.10 GHz. The Savitzky–Golay differentiation routine of Origin 8.0™ was used to numerically obtain these derivatives. The purpose of this figure is to compare the differing features of wild type and R127A in the g_{max} region.

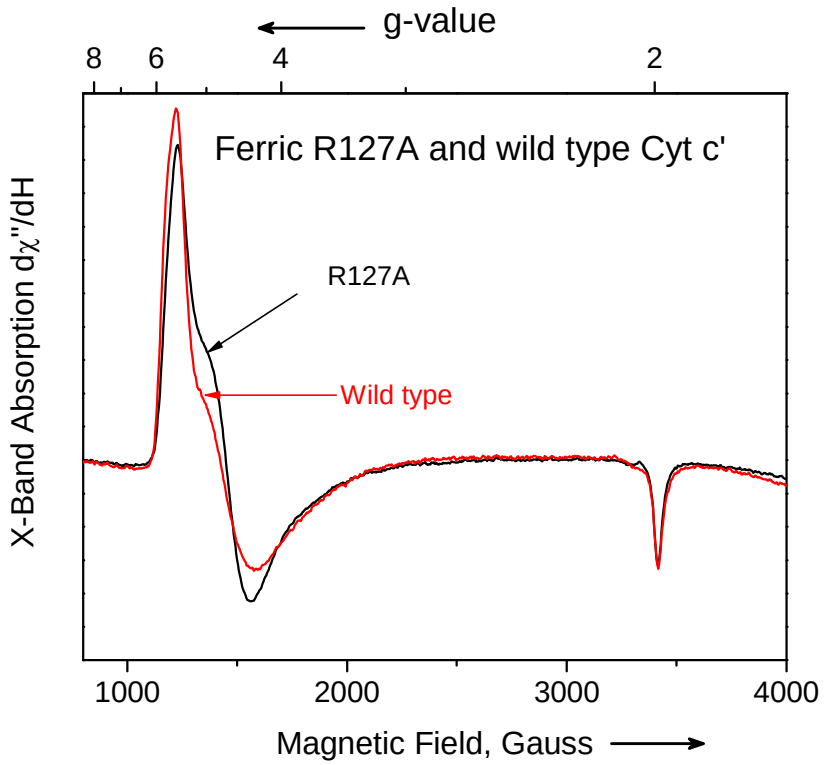


Figure 4S. The purpose of this figure is to show similarity of ***ferric*** heme EPR spectra from wild type and R127A. These spectra were obtained at 15 ºK, 6 Gauss field modulation, 200 s signal averaging over a 4000 Gauss range, 2 mW power, ν_{EPR} = 9.52 GHz. As experimentally estimated at the derivative zero crossing, the $g_{\perp}$ values of two ferric Cyts c′ were respectively 4.8 ± 0.1 and 4.7 ± 0.1 (*6*).

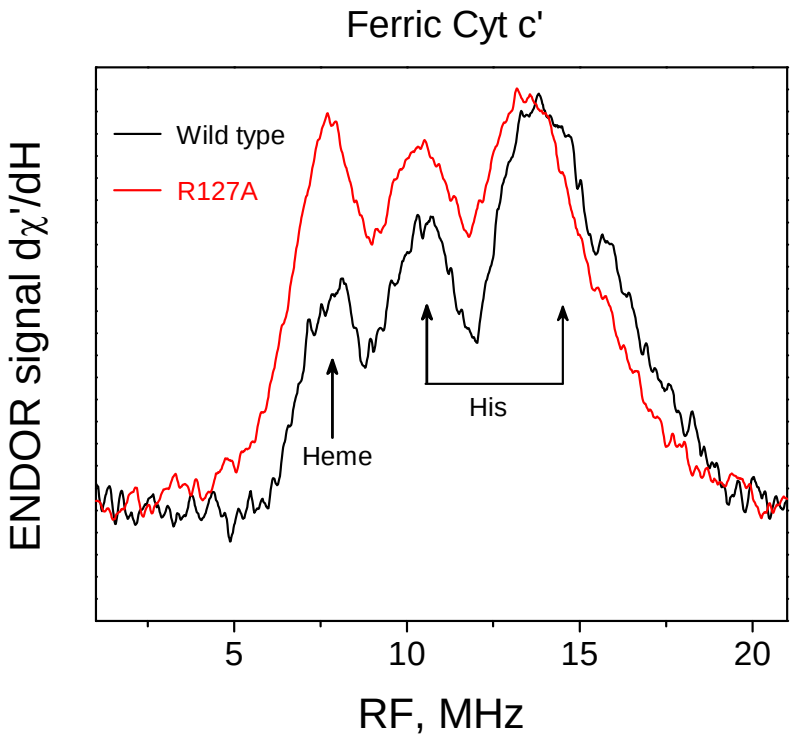


Figure 5S. The purpose of this figure is to show similarity of heme and histidine features from the ***ferric*** heme center of wild type and R127A mutant. Nitrogen ENDOR spectra were collected at H = 12,180 G ($^{14}\nu$ = 3.75 MHz) under adiabatic rapid passage conditions at 2.0 K with ~2.2 G 100 KHz field modulation, ~0.25 μW microwave power, and ~20 W RF (radio frequency) pulsed with a 10μs/90μs duty cycle and swept with a sweep rate of 4 MHz/s.

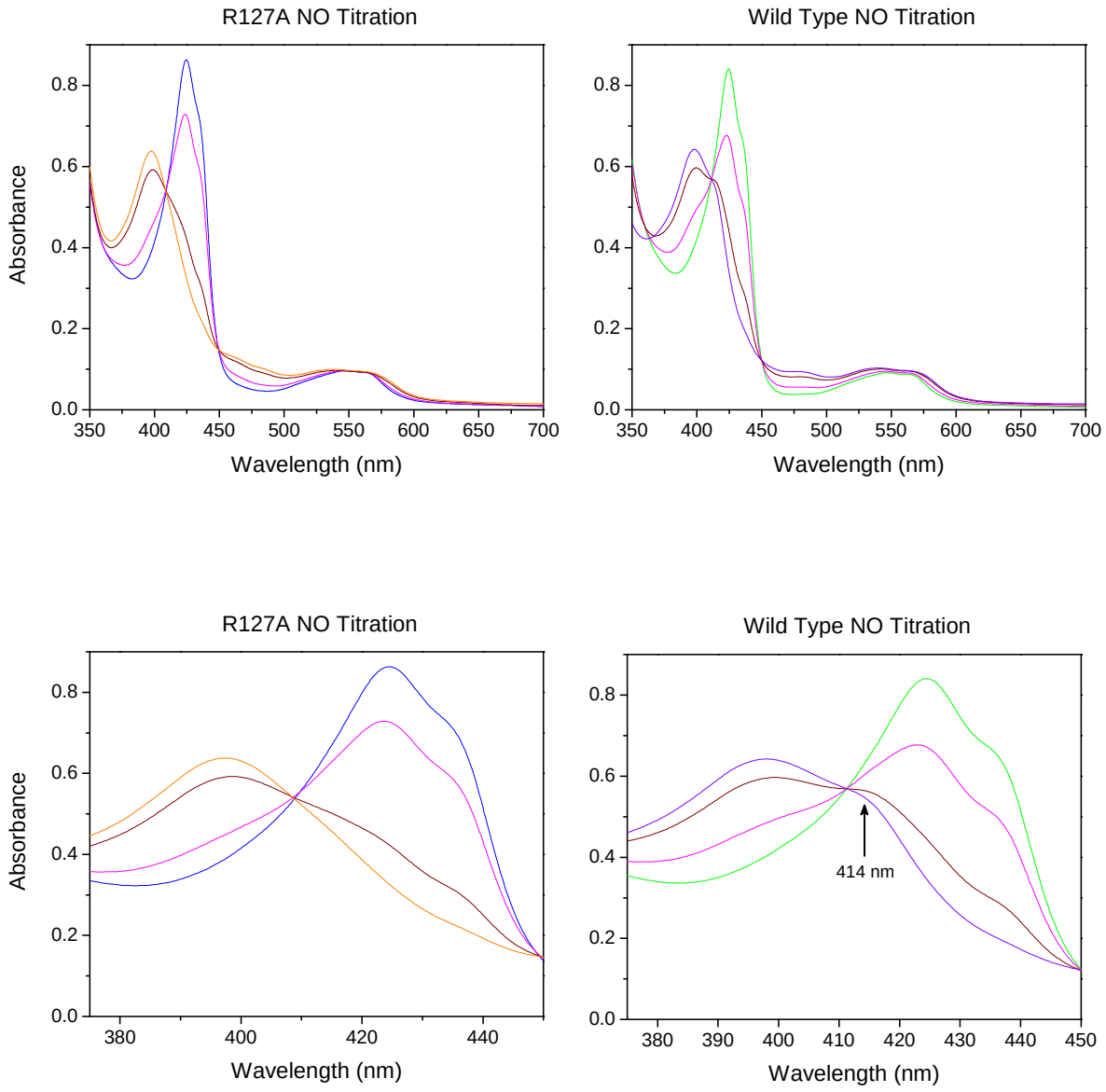


Figure 6S. This figure presents NO titration curves complementary to those in Figure 8 for R127A and wild type Cyt c' overa wider range of wavelengths and in the Soret region. The curves of the top two panels show titrations over the 350-700 nm range, while those on the bottom provide a less crowded compendium and show the presence of a shoulder at 414 nm from the wild type protein. The NO concentrations were as follows: For R127A, [NO] (X 10^{-7} M) 0.41 (blue), 1.13 (magenta), 3.22 (wine), 5.82 (orange). For wild type Cyt c', [NO] (X 10^{-7} M) 1.33 (green), 5.99 (magenta), 14.0 (wine), 23.0 (violet)

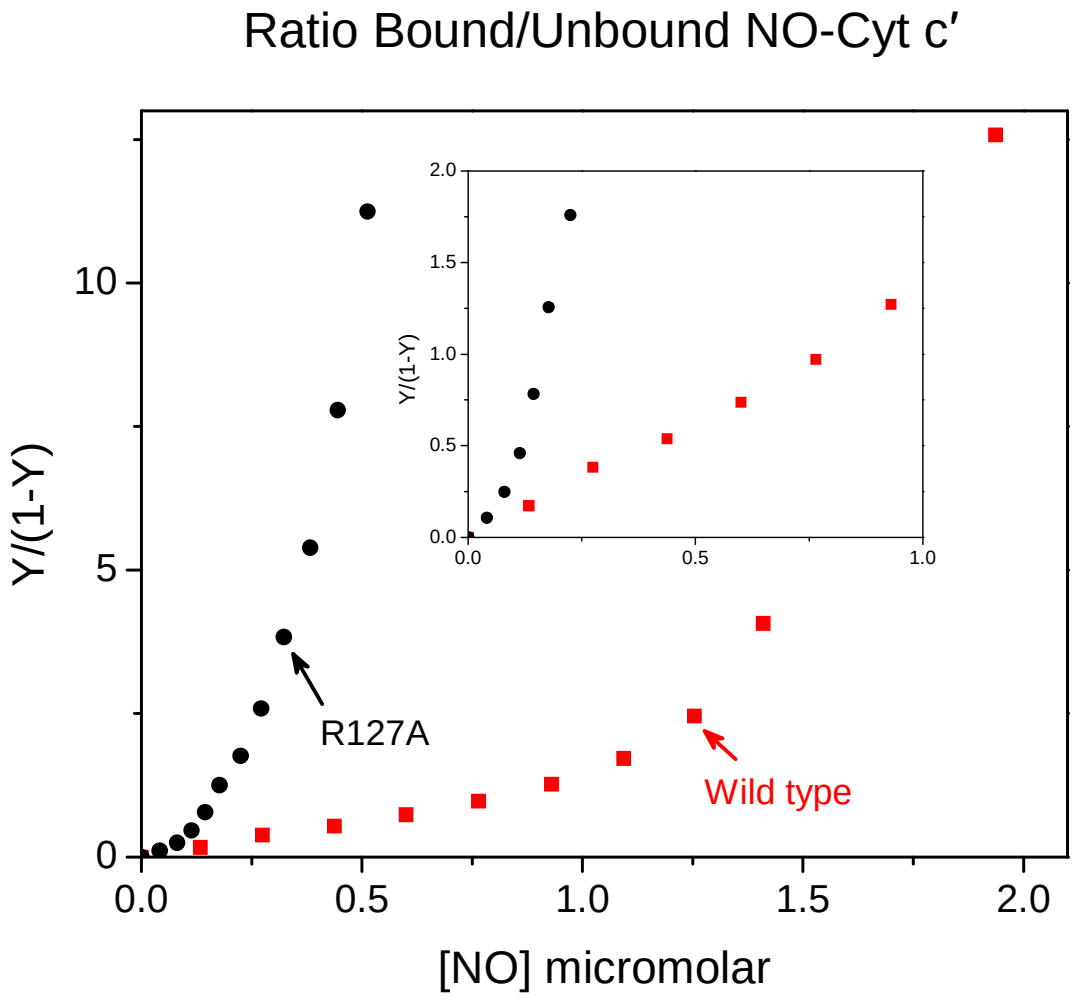


Figure 7S. This figure provides a plot of the ratio of NO-Bound (Y) to NO-Unbound (1-Y) heme as a function of NO concentration for R127A (black) and wild type (red) Cyt c′. Data were taken from **Figures 8** and **9**. The inset expands the region where Y/(1-Y) ≈ 1 (i.e., Y ≈ ½). Non-linear behavior implies cooperativity in NO binding.

References

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