Supporting Information

NMR Investigations of the Rieske Protein from *Thermus thermophilus* Support a Coupled Proton and Electron Transfer Mechanism

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Supplemental materials—Technical details and protocols.

Protein Expression and Labeling. [*U*-^{15}N], [*NA*]-Leu, [*NA*]-His-*Tt*Rp was produced by Origami B (DE3)pLysS cells containing pET17b / *Tt*Rp grown on M9 medium supplemented with 2 g glucose, 50 % of the recommend amount of leucine and histidine (total of 0.23 g/L leucine and 0.1 g/L histidine)[1] was added initially, and the remaining 50 % was added just before induction with IPTG.

[*U*-^{15}N], [*NA*]-Leu-*Tt*Rp was produced by a similar strategy,[1] with the exception that an initial 1 L culture of Origami (DE3) cells containing pET17b / *Tt*Rp was grown in LB medium; the cell pellet from this growth was then transferred to 1 L M9 medium containing 1 g/L $^{15}NH_4Cl$ and 0.23 g/L unlabeled leucine; the induction point was one hour after the transfer. The resulting yield was ~30 mg *Tt*Rp per liter culture.

Protein Purification. A published protocol[2] was used, with modifications. The ammonium sulfate precipitation step was omitted. A stronger anion exchange column (DE53 resin) was used with Tris buffer at pH 7.5 instead of DE52 at pH 8.0. A Mono Q column chromatography step on an FPLC system (ÄKTA) with Tris buffer at pH 7.5 and a 0 M −1 M salt gradient was added before the Superdex 75 (16/60) gel filtration step. Purity and protein concentration were calculated from the UV-VIS absorbance ratio of A_{456nm}/A_{279nm}[2,3] The extinction coefficient was predicted by the ExPASy ProtParam program.[4] The labeling efficiency of the selectively labeled samples was analyzed by ESI MS and MALDI MS, and the molecular weight and purity were checked by trypsin digestive analysis followed by MS/ MS analysis with standards. The labeling patterns were checked by ^{1}H-[^{15}N]-HSQC NMR. All these results were consistent with labeling expectations.

NMR Spectroscopy. Triple-resonance NMR data were collected on a 600 MHz Varian spectrometer (Varian, Fremont, CA) equipped with a cryogenic probe. All other NMR data were collected in a Bruker DMX-500 spectrometer (Bruker BioSpin, Billerica, MA). Four Bruker probes were used: two 5 mm tunable broadband X-[^{1}H] probes, a ^{13}C, ^{15}N-[^{1}H] TXO probe, and a cryogenic ^{13}C, ^{15}N-[^{1}H] TXO probe. Unless otherwise specified, the TXO probe was used for all experiments except for ^{13}C-^{13}C COSY of reduced *Tt*Rp, which used the cryo-TXO probe. 1D ^{1}H NMR spectra were recorded and used for chemical shift referencing. The oxidation state of each sample was monitored by reference to 1D ^{1}H superWEFT (SW) spectra.[5] Two-dimensional ^{1}H-[^{15}N]-HSQC spectra were collected at each pH titration point to verify that the protein was still folded.

NMR Data Processing and Analysis. COSY Spectra were processed with XWIN-NMR software such that the diagonal peaks were in-phase absorptive, and the cross peaks were anti-phase dispersive.[6]

Nitrogen-15 spectra were processed twice, once with standard line broadening (33 or 66 Hz) and once with a large line broadening (500 Hz). The second spectrum was subtracted from the first as a means for baseline correction. Some of the ^{15}N NMR difference spectra were processed further by a user-defined spline (special baseline correction) in XWIN-NMR. Carbon spectra were processed with 33 or 66-Hz line broadening. 2D carbon spectra were zero-filled in the indirect dimension prior to Fourier transformation. 2D-constant time ^{13}C-[^{13}C] COSY-superWEFT (SW) spectra (2D ^{13}C-[^{13}C] CT-COSY-SW)[7,8] data were collected with the following parameters: complex points, 300 (t_1) × 4096 (t_2); spectral width, 300 (t_1) × 300 (t_2) ppm; acquisition time, 55 ms; superWEFT delay, 49 ms; recycling delay, 1 ms; number of scans, 336.

Figure 2 Technical Details. TXO probe was used in (A, D, F, H). The broadband probe was used in (B, C, E, G). NMR parameters: acquisition time, 10.1 ms; recycling delay, 3.5 ms; spectral width, 1000 ppm; carrier frequency, 300 ppm; number of data points, 1024; 90° pulse, 30.5 ms (TXO), 19 ms (broadband); pulse level, 3 dB; receiver gain, 8 × 1024 .

Figure 3 Technical Details. Spectrum (A) was processed with a strong sine square window function, SSB = 6, to gain better resolution. NMR parameters: acquisition time, 48 ms; recycling delay, 1 ms; spectral width, 340 ppm; number of data points, 4×10^3; carbon carrier frequency, 110 ppm; number of scans, 1.9×10^6; 90° pulse length, 6.38 μs; power level, 0 dB; nitrogen carrier frequency, 9846 Hz (oxidized *Tt*Rp), and 21455 Hz (reduced *Tt*Rp), continuous wave decoupler power level, 32.5 dB.

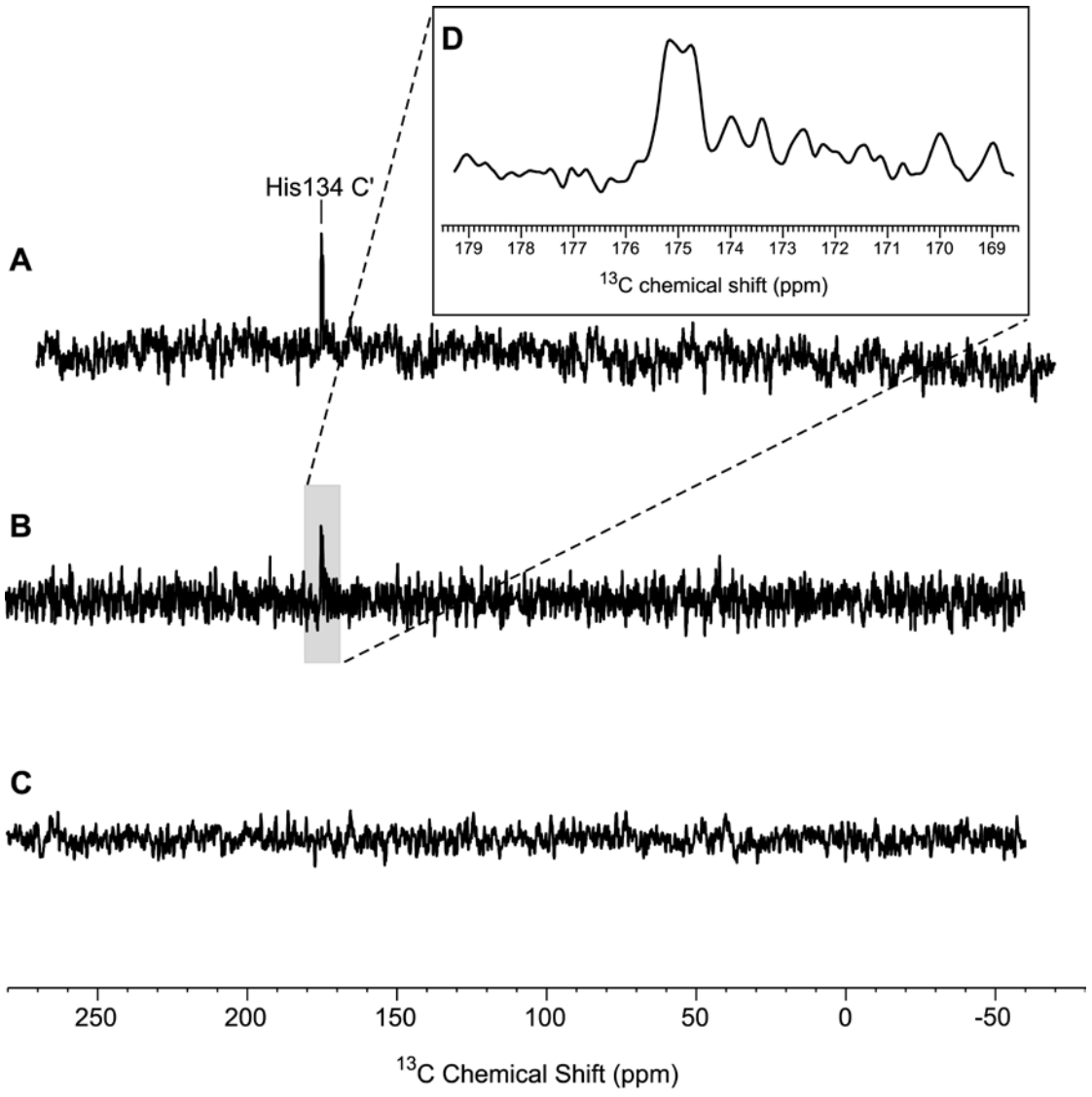

Figure S1. Control double resonance difference decoupling experiments with 21 mM oxidized [*U*-^{13}C, ^{15}N]-His, [^{15}N]-Leu *Tt*Rp. (A) Original settings of the difference decoupling experiment; average of 4.2×10^6 scans. (B) Positive control: 17 mM protein with carrier frequency shifted from 100 ppm to 110 ppm; average of 1.9×10^6 scans. We collected 1D ^{13}C-SW spectra before and after each difference decoupling experiment to make sure that the peak did not move. (C) Negative control: decoupler set to be off-resonance all the time; average of 1.25×10^6 scans. (D) Expansion of scan B (as shown in Figure 3).

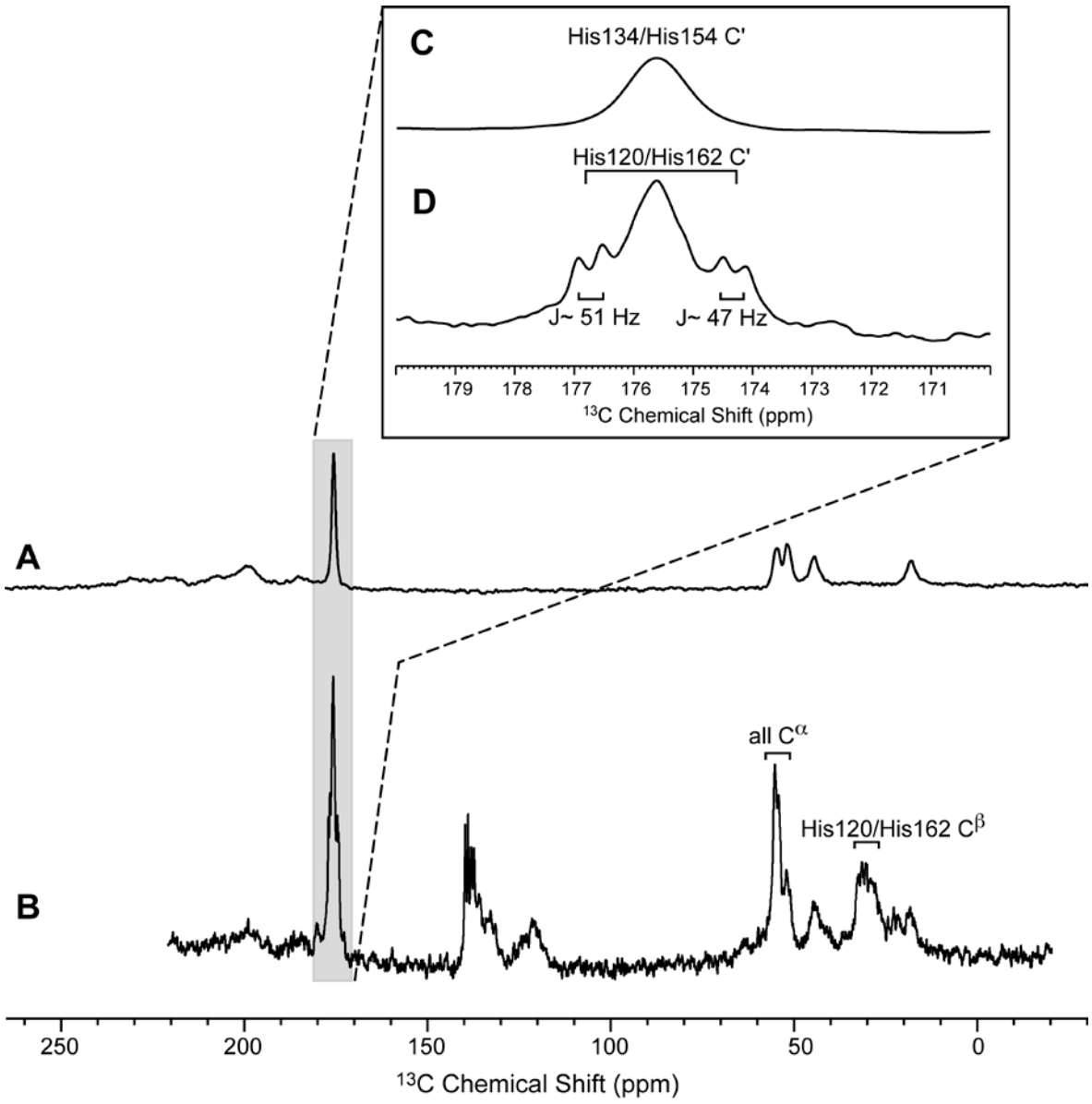

Figure S2. Comparison of 1D ^{13}C-SW spectra of ~20 mM oxidized [*U*-^{13}C, ^{15}N]-His, [^{15}N]-Leu *Tt*Rp. (A) Data collection optimized for paramagnetic signals. (B) Data collection optimized for diamagnetic signals. (C) Expansion of the ^{13}C′ region of trace A showing the absence of signals from the diamagnetic histidines. (D) Expansion of the ^{13}C′ region of trace B showing signals attributed to the two diamagnetic histidines His120 and His162, which exhibit scalar coupling constants of around 50 Hz.

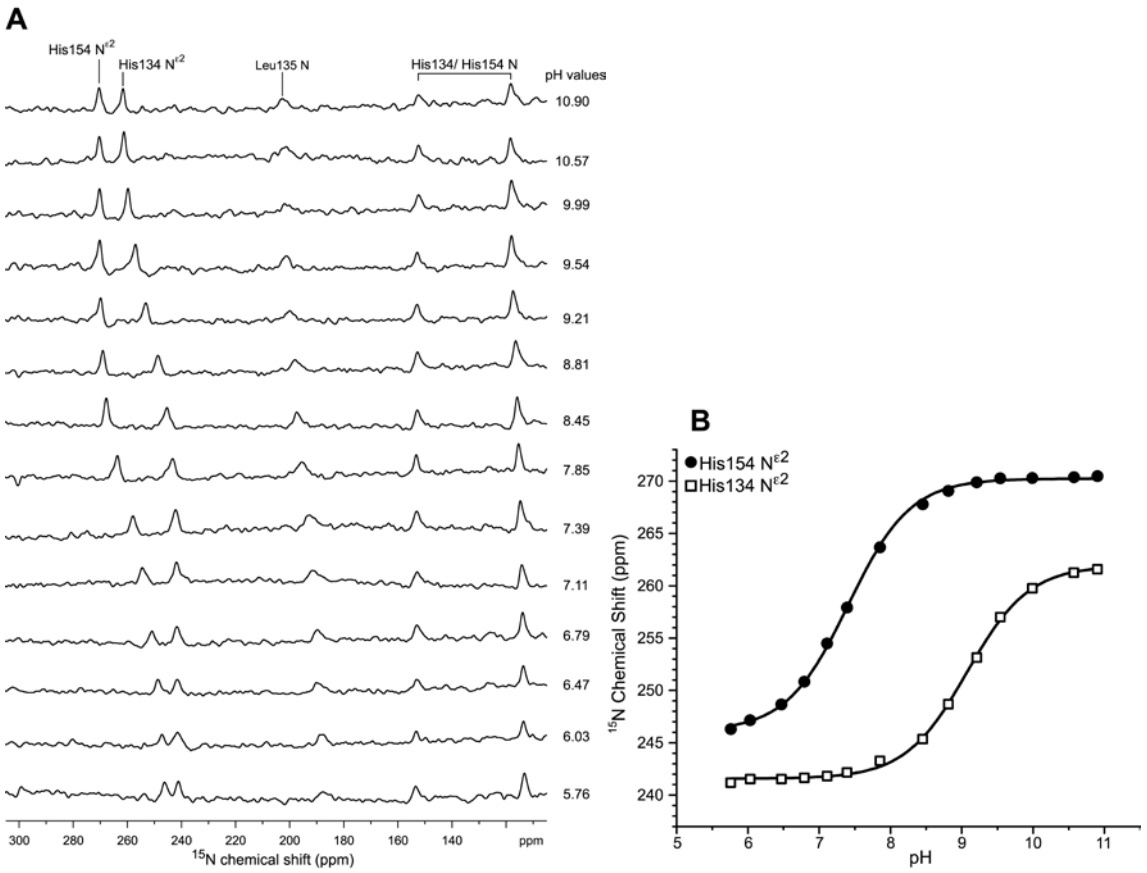

Figure S3. Data from 1D ^{15}N NMR spectra of 21 mM oxidized [*U*-^{13}C, ^{15}N]-His, [^{15}N]-Leu *Tt*Rp collected at 298 K at various pH values. On the basis of data shown in Figures 4 and 5, the lower pK_a value was assigned to His154 and the higher pK_a value was assigned to His134 (Table 1). (**A**) Representative ^{15}N NMR spectra. Peaks assignments are shown over the top trace. (**B**) Chemical shifts of the peaks assigned to the $^{15}N^{\varepsilon 2}$ of His134 (open squares) and the $^{15}N^{\varepsilon 2}$ of His154 (filled circles) as a function of pH. Fitting of the data points with the Hill coefficient fixed at 1.00 (curves) yielded His 134 pK_a = 9.07 ± 0.02 and His154 pK_a = 7.41 ± 0.01.

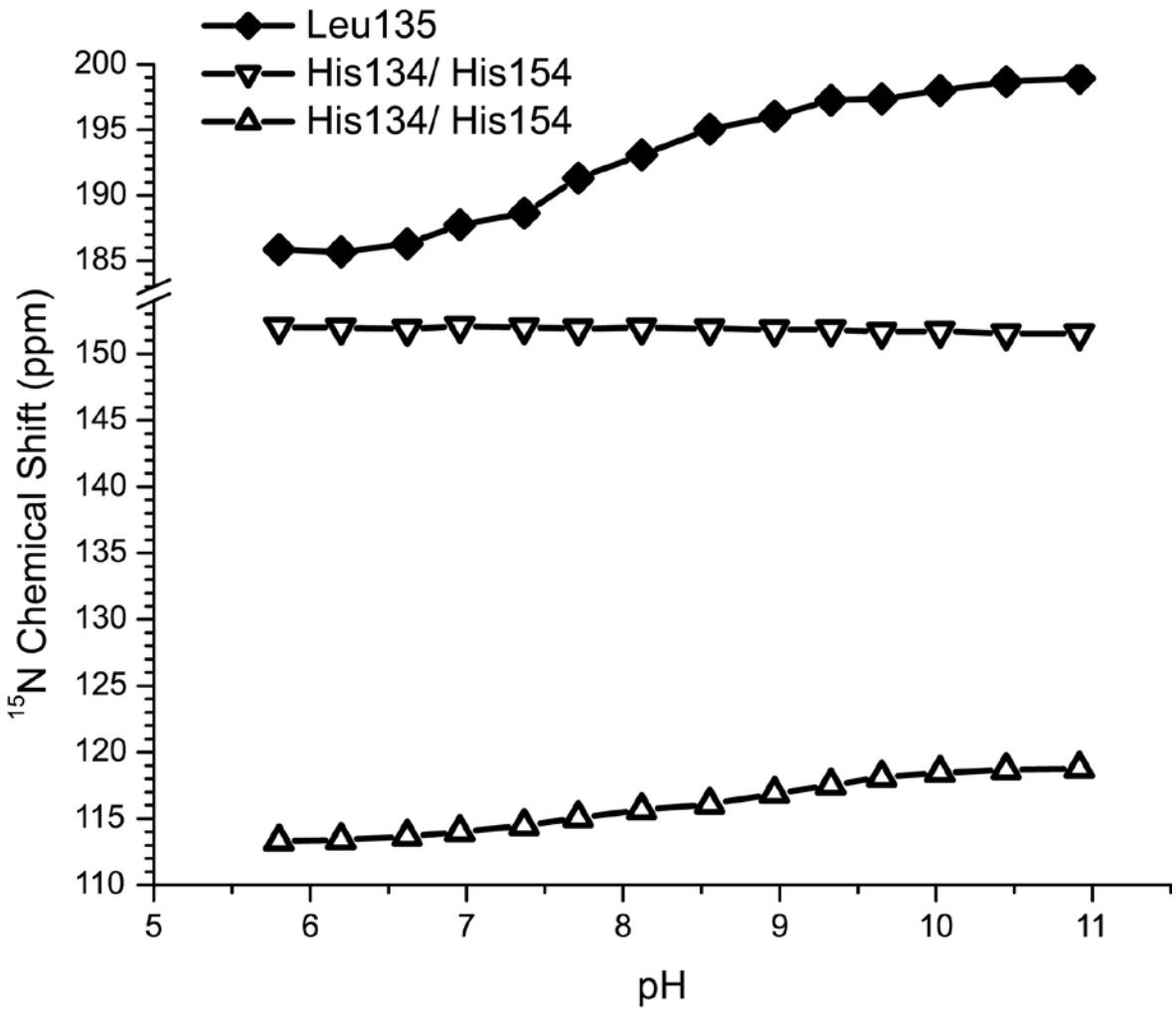

Figure S4. Chemical shifts from 1D ^{15}N NMR spectra of 21 mM oxidized [*U*-^{13}C, ^{15}N]-His, [^{15}N]-Leu *Tt*Rp collected at 298 K at various pH values plotted as a function of pH. The backbone ^{15}N signals from Leu135 N and one of the cluster ligated histidines could be fitted to a single pK_a of 7.81 with a Hill coefficient of 0.49. A titration study of the same labeled protein, but in a solution without ferricyanide, was carried out with data collected at 30 pH values. The latter data set could be fitted simultaneously with Hill coefficients fixed at 1.0 to yield pK_a values of 7.36 (0.08) and 9.34 (0.17) (data not shown).

Supplemental References

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