Supporting Information for:

Characterization of Conformational Exchange of a Histidine Side-chain: Protonation, Rotamerization and Tautomerization of His61 in Plastocyanin from *Anabaena Variabilis*

Mathias A. S. Hass, D. Flemming Hansen, Hans E. M. Christensen, Jens J. Led and Lewis E. Kay

Sample Production:

The ^{2}H-,^{15}N-labeled *A. v*. PCu was produced heterologously in *Escherichia coli* from the previously described strain[1] by fed-batch cultivation. A glycerol stock of D_2O-adopted expression strain[2] was prepared as follows: The expression strain was plated on Luria-Bertini (LB) 100 mg/l ampicillin ager plates. A single colony was inoculated in LB/D_2O medium (LB powder media dissolved in D_2O) and incubated overnight at 37 °C. Subsequently D_2O tolerant cells were diluted 200-fold into LB medium containing 25 mgL^{-1} ampicillin, 2 gL^{-1} glucose and 90 % D_2O, and incubated at 37 °C until an OD_{600} of 1.5. A glycerol stock of D_2O-adopted cells was prepared by mixing 0.5 mL culture with 116 μl 80% glycerol in D_2O. The stock was stored at –80 °C.

The pre-culture for the fermentation was prepared as follows: An aliquot of the D_2O adapted glycerol stock was streaked on a LB/D_2O ager plate containing 25 mg/l ampicillin and 2 g/l glucose. The plate was incubated overnight at 37 °C. A single colony was used to inoculate 25 ml LB/D_2O medium containing 25 mg/l ampicillin and 2 g/l glucose (LB/D_2O/25amp/2G) that was incubated at 37°C and 250 rpm for 11 h. The cells were precipitated by centrifugation (4 C, 3000 rpm, 10 min.) and re-suspended in fresh LB/D_2O/25amp/2G medium. Subsequently 6.5 mL were used to inoculate 650 ml

LB/D_2O/25amp/2G medium. The culture was incubated at 37 °C and 250 rpm for 16 h. Finally the cells were precipitated by centrifugation (4 C, 3000 rpm, 10 min.) and most of the medium was removed by decantation. The remaining 15 ml medium was used to re-suspend the cells and the cell suspension was used to inoculate the fermentor.

A Biostat B fermentor with a 5 l autoclavable jacketed glass vessel (B. Braun Biotech International) equipped with a Mettler Toledo dissolved dioxygen sensor and a Mettler Toledo gel combination pH electrode was used. Prior to autoclaving the pH electrode was calibrated. Fermentation data were collected using the FoxyLogic fermentation Control Program version 4.3. During cultivation, the temperature was maintained at 30 C and the pH at 6.8±0.1 by PID-controlled addition of 1 M NaOH in D_2O. Adjustment of the dioxygen electrode (zero-point and 100% settings) was performed after flushing the fermentor with dinitrogen and a 5 l per minute flow rate of air, respectively, at a stirring speed of 800 rpm. The level of dissolved dioxygen was maintained at 71% by using a double cascade in which the stirring speed was first increased to a maximum of 800 rpm followed by a gasmix-mode where the airflow was enriched with pure dioxygen to maintain the required level of dissolved dioxygen in the medium. The gas flow was maintained at 5 l per minute. Antifoam B (Sigma-Aldrich, A5757), four times diluted in D_2O, was added to a concentration of 20 ppm at the start of the fermentation and then automatically added as required throughout the fermentation.

The initial batch of 2.0 l synthetic medium was made according to the recipe of Cai *et al.*[3] and contained (a) 39.0 g KH_2PO_4, 39.3 g K_2HPO_4 $3H_2O$, 38.8 g Na_2HPO_4 $2H_2O$, 5.87 g Na_2SO_4, 3.00 g NH_4Cl; (b) 6.1 g $MgCl_2$ $6H_2O$; (c) 30 ml trace element stock

solution; (d) 30 ml vitamin solution; (e) 18 ml 5 mg/ml thiamine-HCl; (f) 33 mg/l ampicillin. All components (a) - (f) were dissolved separately in D_2O and filter sterilized. The trace element stock solution contains per 100 mL: 0.50 g Na_2 –EDTA, 0.60 g $CaCl_2$ $2H_2O$, 0.08 g $CoCl_2$ $6H_2O$, 0.12 g $MnCl_2$. $2H_2O$, 0.03 g $CuCl_2$ $2H_2O$, 0.002 g H_3BO_3, 0.070 g $ZnSO_4$ $7H_2O$ and 0.60 g $FeSO_4$ $7H_2O$. The salts were added to 70 ml D_2O in the order given and the pH was adjusted to 5 with 5M NaOH and to 6.5 with 1M NaOH. Finally the volume was adjusted to 100 ml. The vitamin stock solution contains per 100 ml: 10 mg biotin, 10 mg choline chloride, 10 mg folic acid, 10 mg niacinamide, 10 mg calcium D-pantothenate, 10 mg pyridoxal-5-phosphoric acid and 1 mg riboflavin[2]. The pH vitamin solution was adjusted to 7 with 1 M NaOH dissolved in D_2O.

A total of 1 l of feed solution containing 28 g/l glucose in D_2O was exponentially feed to the fermentor over 70 hours. When the glucose was depleted (a pO_2 peak was observed) 0.9 g of $^{15}NH_4Cl$ (300 g/l in D_2O) and 0.8 glucose (400 g/l in D_2O) were added. When a second pO_2 peak was observed 7.2 g glucose was added. When a third pO_2 peak was observed 3.6 g of $N^{15}H_4Cl$ was added and the culture was induced by addition of isopropyl- D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM IPTG. After 3 hours the cells were harvested by centrifugation at 3000 g for 15 minutes at +4 C and washed with 5 mM MES/NaOH pH 6.5. Finally the cells were stored at –80 C until isolation and purification of the protein was initiated. The produced ^2H-,^{15}N-labeled *A.v.* PCu was purified as described previously.[4] Approximately 25 mg per liter of culture of pure PCu with a peak ratio of A_{278}/A_{597} of 1.18 was obtained.

Purified oxidized ^{2}H-,^{15}N-PCu was exchanged into a 10% D_2O 100mM NaCl solution and concentrated to 1 mM by ultrafiltration using a stirred Amicon cell fitted with an YM3 membrane. Reduced ^{2}H-,^{15}N-PCu was obtained by reduction with five-fold molar excess of sodium ascorbate for 1h at +4 C. Excess ascorbate was removed and the protein was exchanged into a 10% D_2O 100 mM NaCl solution and concentrated to 1 mM by ultrafiltration.

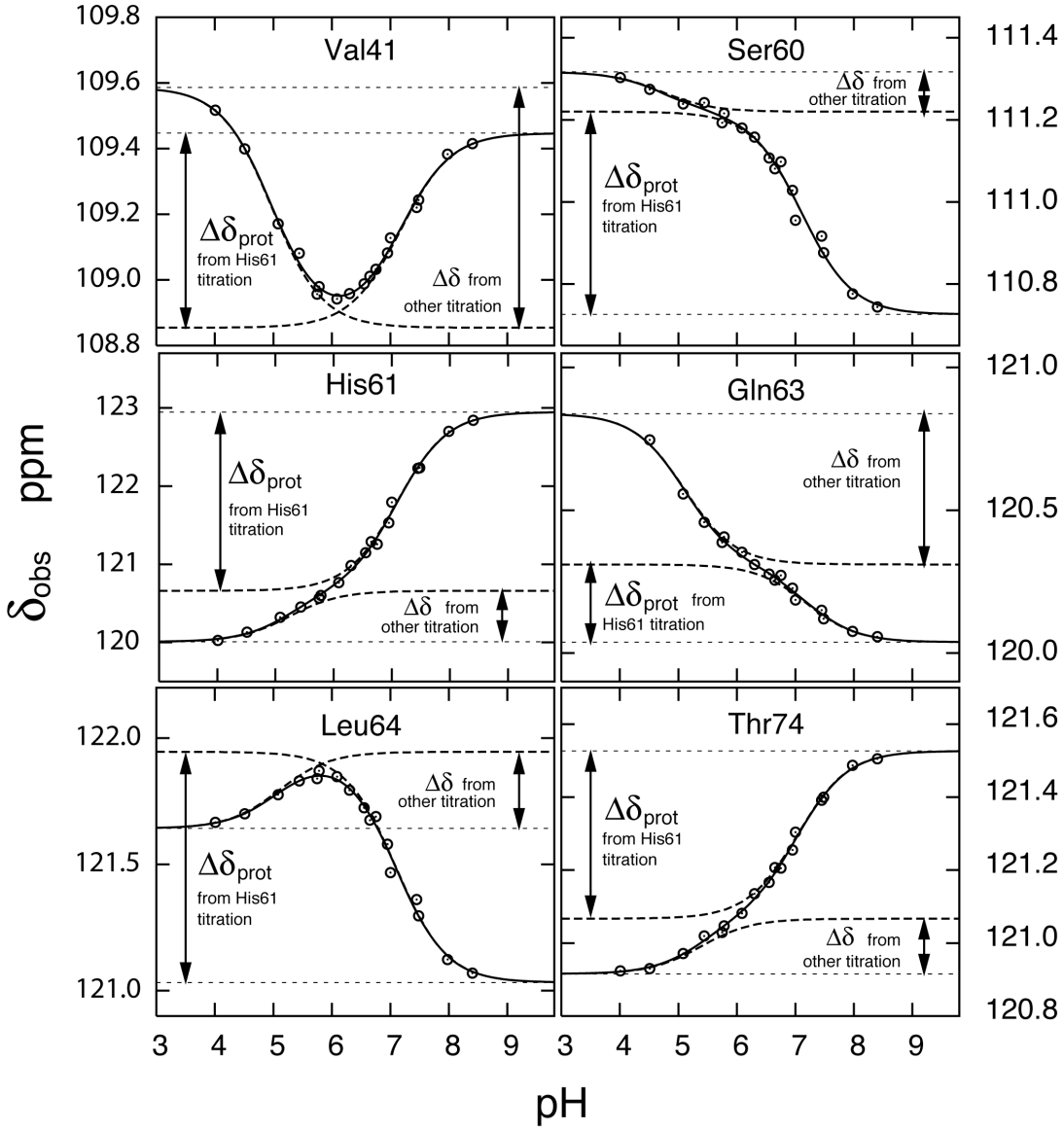

Figure S1. Backbone ^{15}N chemical shift titration curves as a function of pH, used to determine $_{prot}$, for residues Val 41, Ser 60, His 61, Gln 63, Leu 64 and Thr 74 in *A.v.* PCu, 25 C. The chemical shift as function of pH is fit by an equation that includes two independent titrations (solid line; Eq. [11] of the text) where the dotted curves indicate the contributions from each of the two titrations. Values of $_{prot}$, $_{other}$ resulting from the protonation of His 61 and the protonation of other basic groups in the protein, respectively, are indicated by arrows. The titration associated with a pK_a value of 7 corresponds to that of His 61 whereas the titration with midpoint at pH 5 results from other titrating groups in *A.v.* PCu, mainly His 92 and Glu 30. The horizontal lines indicate the chemical shift according to the fit at infinitely high and low pH values.

References

(1) Hass, M. A. S.; Thuesen, M. H.; Christensen, H. E. M.; Led, J. J., *J. Am. Chem. Soc*. **2004**, *126*, 753-765.
(2) Venters, R. A.; Huang, C.-C.; Farmer, B. T.; Trolard, R.; Spicer, L. D.; Fierke, C. A., *J. Biomol. NMR* **1995**, *5*, 339-344.
(3) Cai, M.; Huang, Y.; Sakaguchi, K.; Clore, G. M.; Gronenborn, A. M.; Craigie, R., *J. Biomol. NMR* **1998**, *11*, 97-102.
(4) Schmidt, L.; Christensen, H. E. M.; Harris, P., *Acta Crystallogr., Sect D: Biol. Crystallogr*. **2006**, *D62*, 1022-1029.