Abiotic protein fragmentation by manganese oxide: implications for a mechanism to supply soil biota with oligopeptides.

Patrick N. Reardon[b], Stephany S. Chacon[a], Eric D. Walter[b], Mark E. Bowden[b], Nancy M. Washton[b], Markus Kleber[a,c].*

[a]Department of Crop and Soil Science, Oregon State University, Corvallis OR 97331

[b]Environmental Molecular Science Laboratory, Pacific Northwest National Laboratory, Richland WA 99354

[c]Institut fuer Bodenlandschaftsforschung, Leibniz Zentrum für Agrarlandschaftsforschung (ZALF), Eberswalder Straße 84, 15374 Müncheberg, Germany.

KEYWORDS NMR, EPR, birnessite, kaolinite, protein, peptide, nitrogen.

Supporting Information:

15 pages in total

11 Figures

Supporting Experimental Procedures

The protein Gb1 was expressed in *Escherichia coli* BL21 cells at 37° C in either Luria Broth or modified M9 Minimal media supplemented with $^{15}NH_4Cl$ and ^{13}C-glucose[1]. The cells were grown to an optical density at 600 nm (OD_{600}) of ~0.6 and protein expression was induced with 1mM isopropyl-β-D-thiogalactoside (IPTG) for 6 hours. The cells were pelleted by centrifugation at 6000g for 10 minutes at 4 °C. The cell pellet was suspended in 10 mL of 50 mM $NaPO_4$ buffer at pH 7 containing 10mM phenylmethylsylfonyl floride (PMSF), and 25U of DNAse I. The cells were lysed by incubating the solution with 1 mg of lysozyme at 37° C for 30 minutes, followed by sonication in an ice bath for a total of 3 min. The supernatant from the cell lysis was collected and heated at 80°C for 5 min and centrifuged at 21000g to remove precipitated protein. The Gb1 was subsequently purified by affinity chromatography using an Immunoglobulin G (IgG) column (GE lifesciences). The Gb1 protein was eluted from the column with 0.5 M ammonium acetate buffer at pH 3.4. The fractions containing Gb1 were lyophilized and dissolved in 6 M guanidine HCl, 50 mM sodium phosphate pH 7 and dialyzed into 5 mM Sodium Acetate (pH 5.0), 5 mM Sodium Phosphate (pH 7.0), or water using dialysis cassettes with a 3kDa cut off (Pierce).

Acid birnessite was synthesized from potassium permanganate as previously described [2]. Excess permanganate was removed by extensive dialysis against water. The dialyzed birnessite was dried by lyophilization. The XRD data obtained on our synthesized birnessite is consistent with the results in the literature for "acid birnessite" [2]. The BET surface area of the synthesized birnessite is 39.07 m^2/g, and was determined using the five point Brunauer-Emmett-Teller (BET) method utilizing a Micromeritics ASAP 2020 with N_2 as the adsorbate gas. The average oxidation state of the birnessite was determined using the iodometric method as

previously described [2], except the final concentration of manganese was determined with EPR spectroscopy. The average oxidation state determined by our method is 3.97 +/- 0.03. Ions from the Kaolinite (K-Ga1) were exchanged with Na by suspending the powder in 1M NaCl solution[3]. 25 grams of Kaolinite were mixed with 2.5L of 1M NaCl. The kaolinite suspension was mixed on an orbital shaker for 1 hour before centrifuging it at 2000g for 5 minutes. The supernatant containing the excess Na was decanted and discarded. The pellet was washed by resuspending it in deionized water (2.5L) and shaken for an additional hour. The suspension was centrifuged at 2000g for 5 minutes and the supernatant was decanted. The process was repeated until the discarded supernatant had an electric conductivity below 40 µS. The pellet was lyophilized and stored in brown glass bottles.

We determined the point of zero charge (PZC) of Acid Birnessite and Kaolinite with the Prolonged Salt Titration method [4]. Five mL of distilled deionized water was mixed with 0.25 g of Birnessite or Na-Kaolinite and adjusted with HCl to a pH range of 0.5 to 2.5 for Acid Birnessite and 3.0 to 5.0 for Na-Kaolinite. Every pH value was assayed in triplicate. Samples were equilibrated for 3 days at 25˚C, shaken for 1 hour per day. Samples were centrifuged (11,700g) and the pH values of the supernatant were measured and denoted as pH_i, after which 0.25 ml of 2 M KCl was added to each sample. After 4 hours shaking, samples were centrifuged again and the pH values of the supernatant were measured and denoted as pH_f. A second equilibration of 24 hours (28 hours total) was also carried out and the pH_f values were compared with the ones obtained with the short equilibration time. The measured pH values were corrected for changes in the pH caused by addition of salt as previously described [4]. The ionic activity coefficient used was $f_{0.1} = 0.78$, and was derived from the modified Davies equation. The PZC was obtained from the plot of ΔpH (pH_{f*} - pH_i) against pH_i as the point where ΔpH was equal to

zero. This point was found by fitting the data to a polynomial and determining the appropriate root for the fit equation. The pH measurements were made using an Accumet Basic pH meter (model AB15) (Thermo Fisher Scientific, Waltham, MA). The point of zero charge determined for birnessite is ~1.3. The point of zero charge determined for kaolinite is ~3.8. These values are similar to previously reported points of zero charge for these minerals [4, 5].

Protein was extracted from the minerals using a modified alkaline SDS protocol [6]. The minerals were mixed with 40 μL of Alkaline-SDS buffer (5% SDS. 50 mM Tris-HCl, pH 8.5; 0.15 M NaCl; 0.1mM EDTA; 1 mM $MgCl_2$) for every 20 mg of mineral. The samples were mixed by vortexing for 2 minutes and boiled for 10 minutes. The sample was cooled for 5 minutes at room temperature, vortexed for 2 minutes, and centrifuged at 20,800xg at 4°C for 10 minutes. The supernatants containing the extracted proteins were transferred into 1 mL microcentrifuge tubes. Trichloroacetic acid was added to the supernatant at a final concentration of 25% and stored overnight at 4°C to precipitate the protein. The protein sample was centrifuged at 20,800xg to separate the protein pellet from SDS solution. Protein pellets were washed four times with chilled 100% acetone and recovered by centrifugation at 20,800xg at 4°C. The protein pellet was air-dried and dissolved in 6 M guanidine HCl. Samples were buffer exchanged into the buffer used for adsorption with centrifugal concentrators (GE Lifesciences) and used for NMR analysis. Only samples from kaolinite at pH 5 yielded protein pellets and resonances in the ^{15}N-HSQC spectrum.

Samples from the reaction supernatants were mixed 1:1 with 2X SDS-PAGE gel loading buffer and boiled for 5 minutes followed by separation on a 16% Tris-Tricine SDS gel[7]. The gel was cast and run using a BioRad mini protean 3 gel apparatus at 120 volts. Spectra BR broad

range ladder (Fermentas) was used as a molecular weight marker. The gel was stained using Gel Code Blue (Pierce) according to the manufacturer's instructions.

NMR data were processed with the nmrPipe software package[8]. Polynomial based water suppression was applied to the time domain data. A shifted sine bell windowing function was applied and the data were zero filled to twice the original size prior to fourier transformation in each dimension. Polynomial based baseline correction was applied as the last step of processing.

X-ray diffraction patterns were collected using Bragg-Brentano geometry with either a Panalytical X'Pert or Rigaku Miniflex II diffractometer. Both instruments were equipped with Cu radiation (λ = 1.54059 Å) and a post-diffraction monochromator, and data were recorded using continuous symmetric θ-2θ scans from 5 to 100 °2θ. XRD analysis was conducted on samples of the synthesized "acid birnessite". The observed diffraction pattern is similar to the pattern previously presented for "acid birnessite" [2]. Additional XRD analysis was carried out on birnessite reacted with and without Gb1 protein in water at pH 5 for 24 hours. The XRD pattern observed is nearly identical between the two samples. These XRD results indicate that little modification of the bulk birnessite occurred over the course of 24 hours. This data is shown in Figure S9.

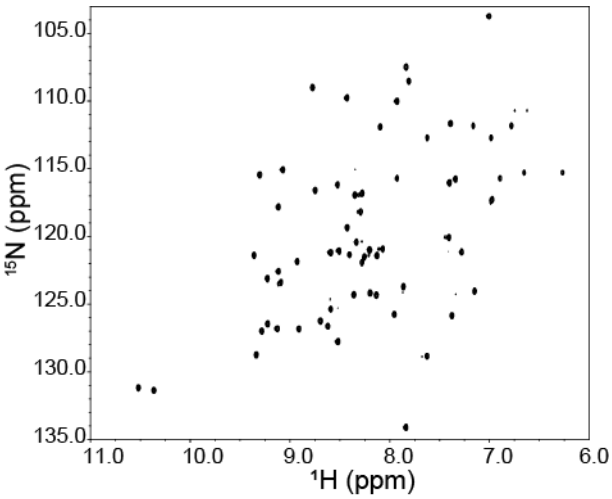

Figure S1. ^{15}N-HSQC spectrum of protein extracted from kaolinite at pH 5.

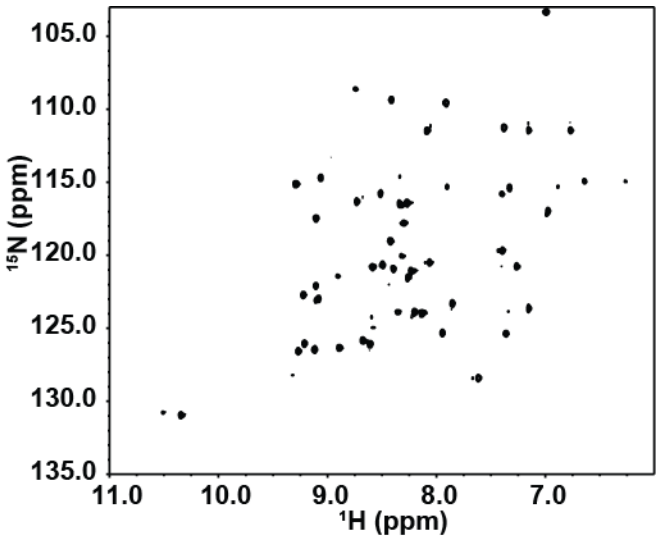

Figure S2. ^{15}N-HSQC spectrum of Gb1 protein after 24 hours in 200 µM $MnCl_2$. The molar concentration of $MnCl_2$ used is ~3 fold higher than that of GB1 and is much greater than the concentration of Mn(II) observable by EPR. This concentration is sufficiently high, based on EPR analysis, to detect possible chemical reactions between Mn(II) and Gb1 that would occur under our reaction conditions, without inducing substantial paramagnetic line broadening that

would prevent observation of the Gb1 resonances. This is not to say that Mn(II) does not interact with the protein. Like most proteins, Gb1 contains exposed negatively charged patches which are likely to interact with Mn(II) at high concentrations and is the likely cause of the modest variation in line broadening observed in this spectrum [9].

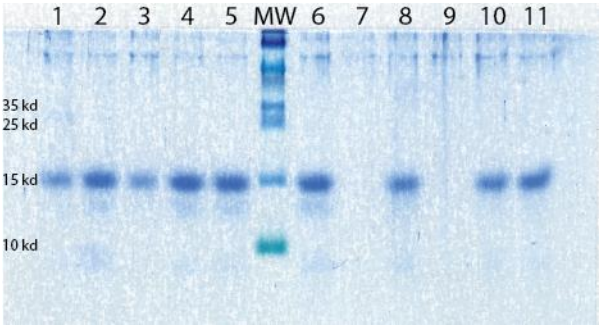

Figure S3. Tris-Tricine SDS-PAGE gel of protein mineral reaction supernatants. Lanes 1-5 are Gb1 added to Kaolinite in various buffer conditions. MW is the molecular weight marker. Lane 6 is the unreacted Gb1 standard. Lanes 7-11 are Gb1 added to Birnessite in various buffer conditions. The buffer conditions are sodium acetate pH 5 (1,7), sodium phosphate pH 7 (2,8) , no buffer pH 5 (3,9), no buffer pH 7 (4,10), and MOPS pH 7 (5,11). GB1 often retains sufficient secondary structure in Tris-Tricine gels to alter its mobility relative to molecular weight standards, causing it to run larger than expected on the gel. The masses for the molecular weight standards are indicated on the left side of the gel.

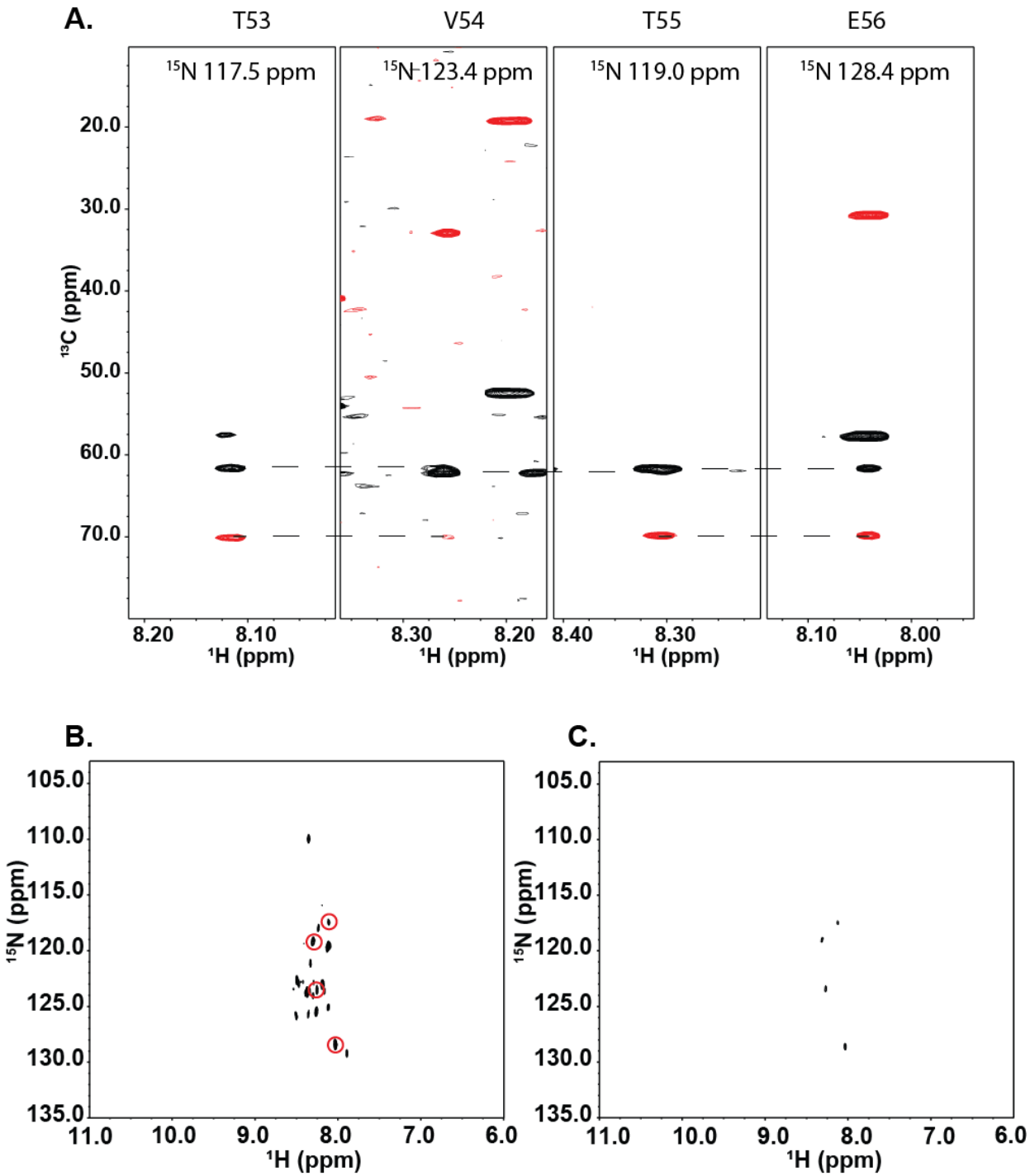

Figure S4. NMR data used for assignment of the first peptide released during birnessite-gb1 reaction. A. Strip plot from the HNCACB used to make the sequential assignments of the peptide. Dashed lines indicate the resonances that are produced by the same carbon nucleus and neighboring backbone amides and were used to make the sequential assignments. Amino acid

types were identified by their characteristic carbon chemical shifts[10]. B. 3D HNCO spectrum collected following a 24 hour reaction between birnessite and Gb1. Data is shown as a projection of the 3D data on to the proton nitrogen plane. Resonances corresponding to the backbone amides of the first peptide released from the birnessite are indicated with red circles. C. ^{15}N-HSQC spectrum collected after a 0.5 hour reaction of birnessite with Gb1 for comparison to B.

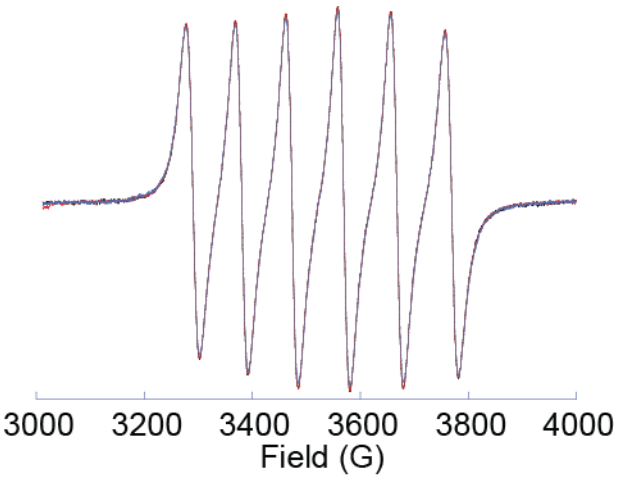

Figure S5. Comparison of EPR spectra of 200 µM $MnCl_2$ under various conditions. The Red spectrum is 200 µM $MnCl_2$ with 0.4 mg/ml Gb1 protein immediately following mixing. The Blue spectrum is the same sample used for the Red spectrum after incubation for 24 hours at room temperature. The Black spectrum is 200 µM $MnCl_2$ without protein. All samples were adjusted to pH 5 prior to analysis. The three spectra are very similar and overlap nearly perfectly.

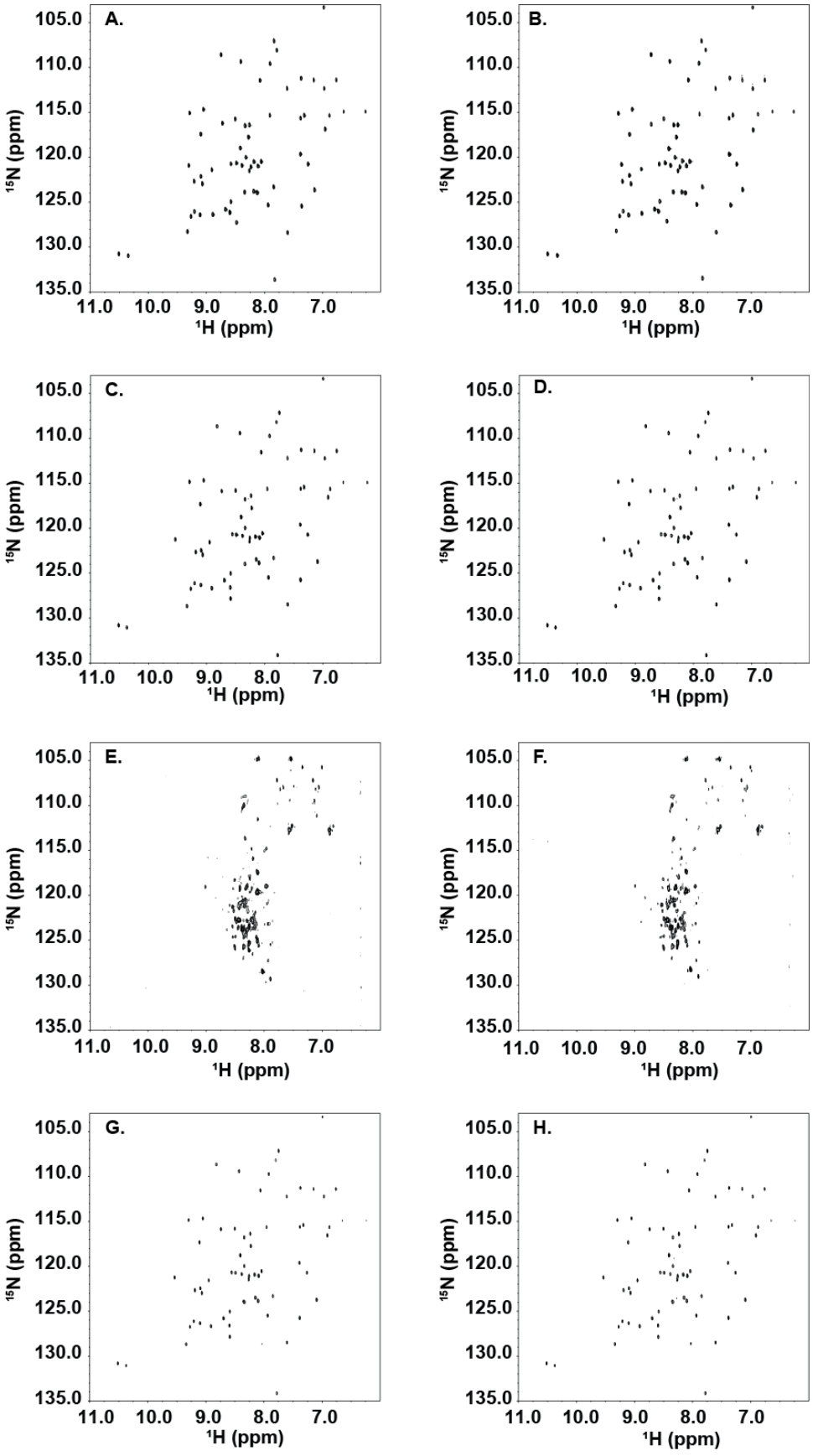
A.
B.
C.
D.
E.
F.
G.
H.
105.0
110.0
115.0
120.0
125.0
130.0
135.0
^{15}N (ppm)
11.0
10.0
9.0
8.0
7.0
^{1}H (ppm)

Figure S6. Comparison of NMR spectra from Gb1 reacted with birnessite or kaolinite for 24 hours in various buffers. A. Kaolinite and no buffer at pH 5. B. Kaolinite and 5 mM Sodium Acetate buffer at pH 5. C. Kaolinite and no buffer at pH 7. D. Kaolinite and 5 mM Sodium Phosphate at pH 7. E. Birnessite and no buffer at pH 5. F. Birnessite and Sodium Acetate buffer at pH 5. G. Birnessite and no buffer at pH 7. H. Birnessite and Sodium Phosphate at pH 7.

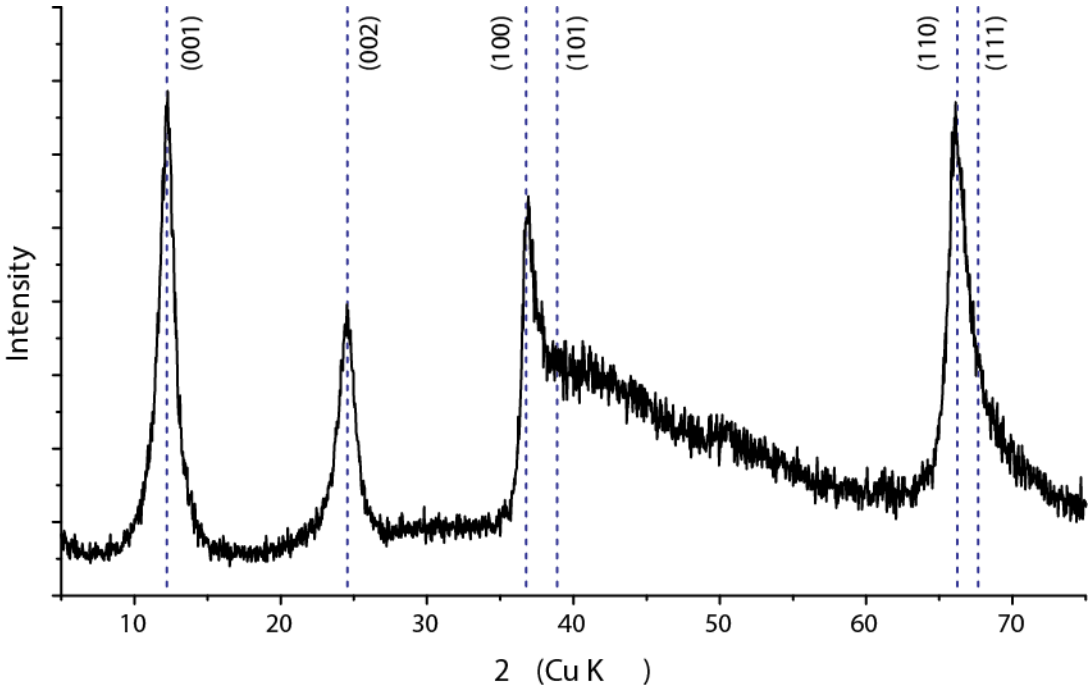

Figure S7. X-ray Diffraction pattern of synthesized acid birnessite. The dashed vertical lines indicate the positions of the peaks expected for a turbostratic birnessite with hexagonal cell parameters a = 2.819 Å, c = 7.244 Å [11]. The pattern bears a strong resemblance to the previously published diffraction pattern of acid birnessite [2].

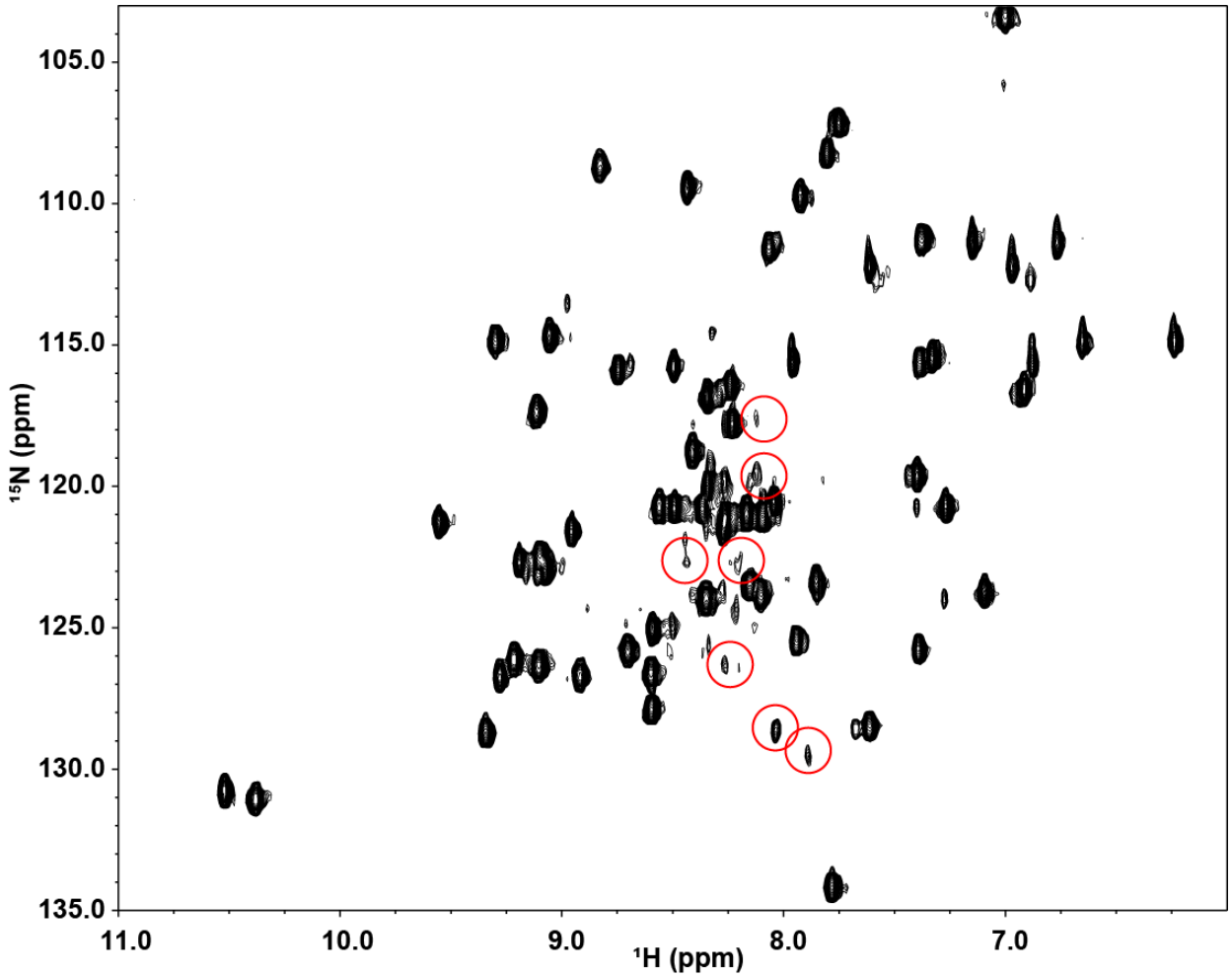

Figure S8. ^{15}N-HSQC spectrum of the birnessite-Gb1 reaction at pH 7 after 72 hours. The contour is set to better show the product peaks. Some of these product peaks are marked by the red circles.

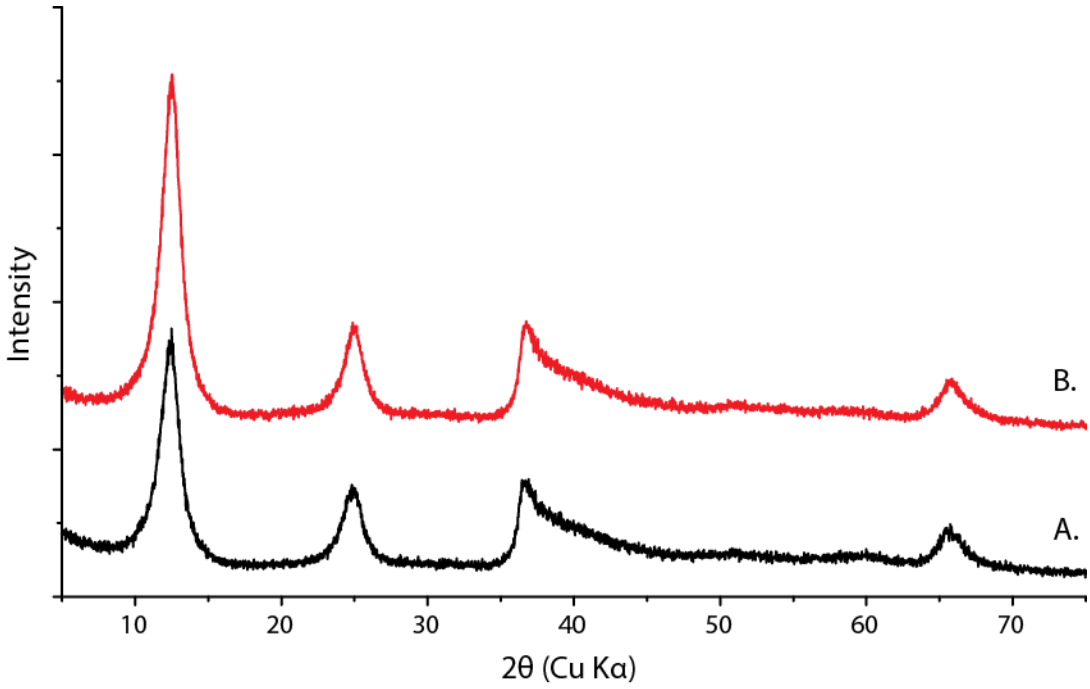

Figure S9. XRD of birnessite reacted with (A.) or without (B.) Gb1.

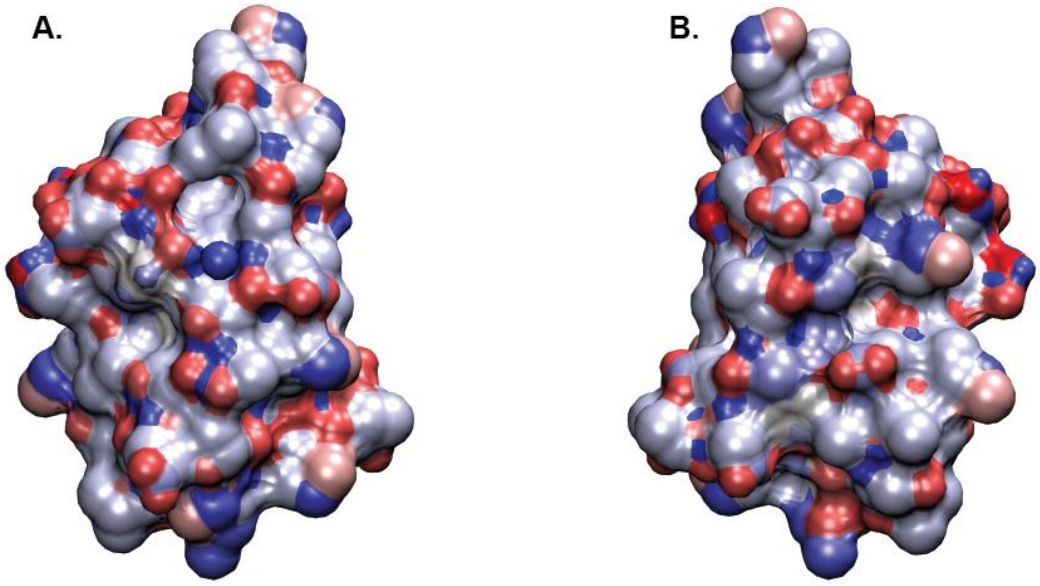

Figure S10. Surface representation of Gb1 with Poisson-Boltzmann electrostatic potential. Positve potential is shown in blue while negative potential is shown in red. A. is rotated 180° compared to B.

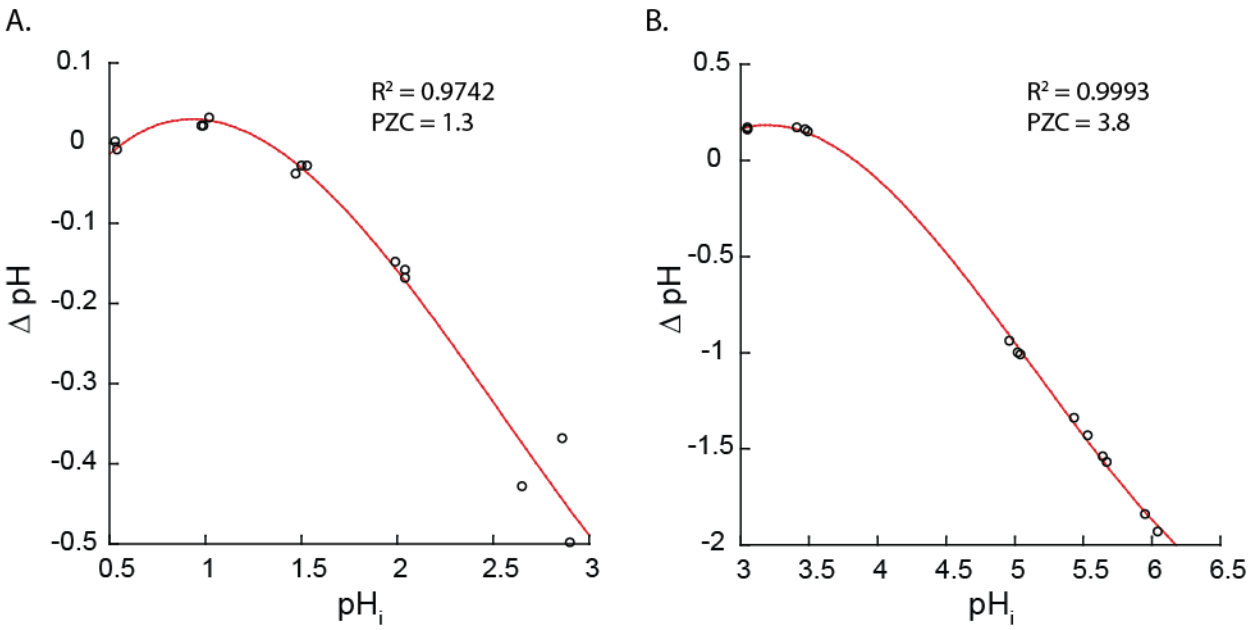

Figure S11. Point of zero charge measurements using the prolonged salt titration method. A. Birnessite point of zero charge. B. Kaolinite point of zero charge.

1. Reardon, P. N.; Spicer, L. D., Multidimensional NMR spectroscopy for protein characterization and assignment inside cells. *J. Am. Chem. Soc.* **2005,** *127*, (31), 10848-9.
2. Villalobos, M.; Toner, B.; Bargar, J.; Sposito, G., Characterization of the manganese oxide produced by Pseudomonas putida strain MnB1. *Geochim. Cosmochim. Acta* **2003,** *67*, (14), 2649-2662.
3. Soukup, D. A.; Buck, B. J.; Harris, W., *Preparing soils for mineralogical analyses*. Soil Science Society of America: Madison, 2008.
4. Tan, W. F.; Lu, S. J.; Liu, F.; Feng, X. H.; He, J. Z.; Koopall, L. K., Determination of the point-of-zero, charge of manganese oxides with different methods including an improved salt titration method. *Soil Science* **2008,** *173*, (4), 277-286.
5. Schroth, B. K.; Sposito, G., Surface charge properties of kaolinite. *Clays Clay Miner.* **1997,** *45*, (1), 85-91.
6. Chourey, K.; Jansson, J.; VerBerkmoes, N.; Shah, M.; Chavarria, K. L.; Tom, L. M.; Brodie, E. L.; Hettich, R. L., Direct cellular lysis/protein extraction protocol for soil metaproteomics. *J Proteome Res* **2010,** *9*, (12), 6615-22.
7. Schagger, H.; von Jagow, G., Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **1987,** *166*, (2), 368-79.

8. Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A., NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR* **1995,** *6*, (3), 277-93.
9. Hocking, H. G.; Zangger, K.; Madl, T., Studying the structure and dynamics of biomolecules by using soluble paramagnetic probes. *Chemphyschem* **2013,** *14*, (13), 3082-94.
10. Wishart, D. S.; Case, D. A., Use of chemical shifts in macromolecular structure determination. *Methods Enzymol.* **2001,** *338*, 3-34.
11. Drits, V. A.; Silvester, E.; Gorshkov, A. I.; Manceau, A., Structure of synthetic monoclinic Na-rich birnessite and hexagonal birnessite .1. Results from X-ray diffraction and selected-area electron diffraction. *Am. Mineral.* **1997,** *82*, (9-10), 946-961.