Electronic Supplementary Material (ESI) for ChemComm.
This journal is © The Royal Society of Chemistry 2015

Electronic Supplementary Information

Probing the nitrite and nitric oxide reductase activity of *cbb*$_3$ oxidase: Resonance Raman detection of a six-coordinate ferrous heme-nitrosyl species in the binuclear *b*$_3$/Cu$_B$ center

Andreas Loullis and Eftychia Pinakoulaki*

University of Cyprus, Department of Chemistry, PO Box 20537, 1678 Nicosia, Cyprus.
**E-mail: effiep@ucy.ac.cy*

Experimental Procedures

The *cbb*$_3$-oxidase was isolated from *P. stutzeri* according to the published procedures (A. Urbani, S. Gemeinhardt, A. Warne and M. Saraste, *FEBS Lett.*, 2001, **508**, 29-35). The purified enzyme was concentrated to 200-400 µM, frozen and stored in liquid nitrogen until use. Chemicals were purchased from Sigma Aldrich and were of analytical grade. The *cbb*$_3$-oxidase samples used for RR measurements were diluted to 50 µM using the desired buffers (pH 7.5, Tris-HCl; pH 9.0, CHES). The isotopically labeled $Na^{15}NO_2$ (98% ^{15}N, 95% CP), $Na^{15}N^{18}O_2$ (98% ^{15}N, 90% ^{18}O, 95% CP) and ^{14}NO gas were obtained from Sigma Aldrich. The isotopically labeled ^{15}NO was produced by addition of dithionite to $Na^{15}NO_2$ solution under anaerobic conditions.

Resonance Raman experiments. For the experiments using nitrite as the substrate, samples of *cbb*$_3$ oxidase were reduced by 300-fold excess of cysteine under anaerobic conditions that were attained by cycling the samples 4-6 times between vacuum and nitrogen in a vacuum line, followed by addition of nitrite solution to a final concentration of 25 mM. It should be noted that under our experimental conditions we do not observe any reduction of nitrite by cysteine in the absence of the enzyme, as also described in previous studies (J. Yi, J. Heinecke, H. Tan, P. C. Ford and G. B. Richter-Addo, *J. Am. Chem. Soc.*, 2009, **131**, 18119-18128, and ref. 10). For the experiments using NO as the substrate, *cbb*$_3$ oxidase samples were reduced by excess dithionite under anaerobic conditions and NO was subsequently added. Resonance Raman spectra were acquired at room temperature (20 ^{o}C) using a 640mm focal length Czerny-Turner spectrograph (Horiba, T64000 system operated in single stage), equipped with a 1800 g/mm holographic grating and a Horiba Symphony BIUV1024x256 CCD detector. Samples were placed anaerobically in a quartz spinning tube to minimize local heating, and scattering was collected in a 90° geometry. The excitation wavelength at 441.6 nm was provided by a Kimmon HeCd laser, and a Semrock 442 nm long-pass edge filter was used to reject Rayleigh scattering. The power incident on the sample was 4 mW and the total accumulation time for each spectrum was 20 min. An Ondax SureLock LM-405 laser with an integrated CleanLine ASE filter was used to provide the excitation wavelength at 405 nm, and a Semrock StopLine 405 nm single-notch filter was

used to reject Rayleigh scattering. The power incident on the sample was 0.5 mW and total accumulation time was 20 min. The Raman shifts were calibrated using toluene. Origin software (OriginLabs) was used for spectra processing and analysis.

FTIR experiments. For the FTIR experiments 200 μM *cbb*$_3$ oxidase samples were reduced by 100-fold excess of cysteine, nitrite solution was added to a final concentration of 6 mM and the samples were placed anaerobically in the FTIR cell comprising of two CaF_2 windows and a 25 μM spacer. FTIR spectra were recorded with 4 cm^{-1} spectral resolution on a Bruker Vertex 70 spectrometer equipped with a MCT detector using the buffer as the background. In the control FTIR experiment (incubation of NO_2^- with cysteine in the absence of *cbb*$_3$ oxidase) N_2O formation was not observed.

Supplementary Figures

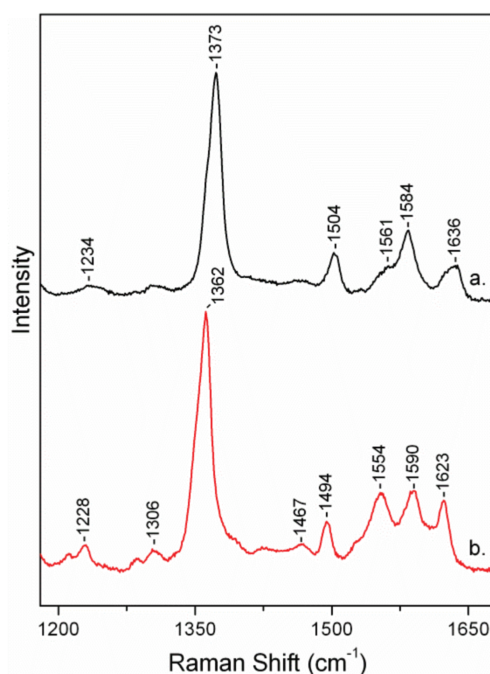

Figure S1: High frequency resonance Raman spectra of oxidized (trace a) and cysteine-reduced (trace b) *cbb*$_3$ oxidase. The excitation used was 405 nm for the oxidized *cbb*$_3$ oxidase and 441 nm for the reduced enzyme.

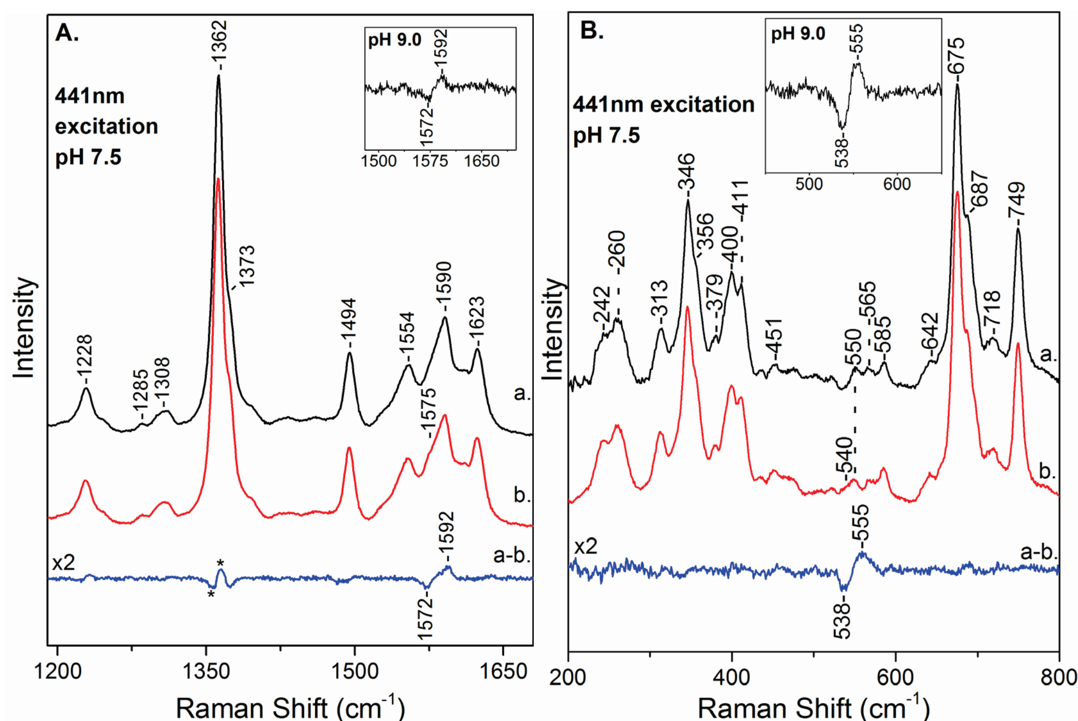

Figure S2: High frequency (panel A) and low frequency (panel B) resonance Raman spectra of the *cbb*$_3$ oxidase adducts formed after addition of $^{14}N^{16}O$ (trace a) and $^{15}N^{16}O$(trace b) to the dithionite-reduced enzyme at pH 7.5. The difference a-b (*cbb*$_3$-$^{14}N^{16}O$ *minus cbb*$_3$-$^{15}N^{16}O$) resonance Raman spectrum multiplied by a factor of 2 is included in each panel. The corresponding difference spectrum at pH 9 is included in the inset in each panel. The excitation wavelength was 441 nm and the power incident on the sample was 4 mW.

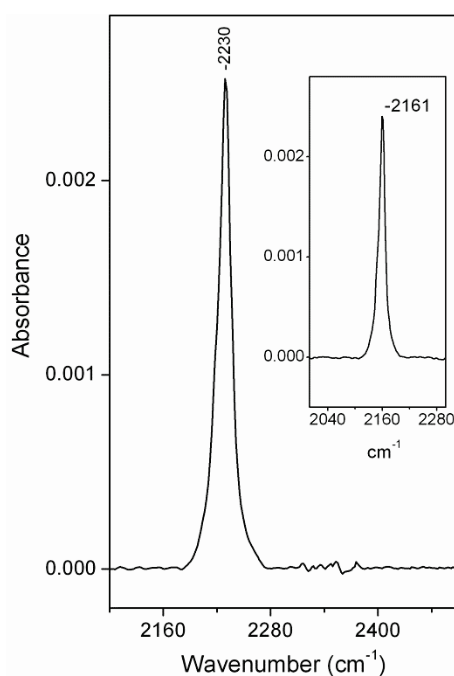

Figure S3. FTIR spectra of the reaction of cysteine-reduced *cbb*$_3$ oxidase with $^{14}NO_2^-$ and $^{15}NO_2^-$ (inset) after 40 min incubation.