**Supporting Information**

**The Role of Hydration on Allosteric Effect: Energy Flow in Hemoglobin**

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**Chemicals**

Hemin (BioXtra, from Porcine, ≥98.0% HPLC) and Hemoglobin from bovine blood (lyophilized powder) have been received from Sigma-Aldrich, and used as received. D2O (99.9% D, Cambridge Isotope Laboratories, Inc.) and ethylene glycol (Fisher Scientific, less 0.03% water) were used as solvents for preparing Hemin and Hemoglobin aqueous solution. Potassium cyanide (Fisher Scientific, 99.3%) and imidazole (Alfa Aesar, 99%) were used as external ligands of Hemoglobin.

**Femtosecond Time-Resolved Infrared Measurement**

Time-resolved Infrared (TRIR) absorption spectra are collected using femtosecond VIS pump-IR probe technique. TRIR system was described in our previous publications.[1](#_ENREF_1),[2](#_ENREF_2) Briefly, TRIR system is introduced as fellow: 800 nm (1 kHz, 30 fs) is generated from Femtosecond Ti:sapphire regenerative amplifier (Legend Elite, Coherent) seeded by a femtosecond Ti:sapphire mode-locked oscillator (Mantis, Coherent). The Legend output power of 3.6 W is split into a 1:1 ratio and sent into two OPA systems (OPerA Solo, Coherent) used to generate UV-vis and IR laser beam, respectively. Excitation wavelength is 400 nm generated from 800nm, and pump beam is delayed by translation stage (Newmark System, Inc.). The IR beam generated by DFG crystal is separated into probe and reference beam by CaF2 beamsplitter (50/50 ± 10% R/T, 2-8 m, ISP Optics), then sent into ImagIR infrared camera (HgCdTe, 2-10 m, 128x128, Santa Barbara Focalplane). The data acquisition is achieved using LabVIEW (National Instruments).

For TRIR measurement, the sample solution is flowed by fluid metering RHSY lab pump (Scientific Support Inc.) through a demountable liquid flow cell with swagelok fittings (DSC-S25, Harrick Scietific Product Inc.). The path length is 80 μm, which is created by Teflon spacer between two polished circular CaF2 windows (25×2 mm, Koch Crystal Finishing, Inc.). 0.6mM hemoglobin and 2.5mM hemin are dissolved into D2O for TRIR measurements. 0.6mM hemoglobin-imidazole and hemoglobin-CN complex were prepared by dissolving 100mM imidazole and 100mM KCN into 0.6mM hemoglobin/D2O solution.

**FT-IR and UV-vis Measurement**

2.5mM Hemin and 0.6mM Hemoglobin aqueous solution created by Teflon spacer (60 μm) between two polished rectangular CaF2 windows (38.5 x 19.5 x 4 mm, Koch Crystal Finishing, Inc.) have been used for FT-IR and UV-vis measurement in DIR Amalgamated Sealed Cells (McCarthy Scientific Co.). Steady state and temperature-dependent FT-IR spectra were collected using Varian 660 IR Spectrometer while steady state UV-vis spectra were obtained using a Lambda35 Spectrophotometer (Perkin Elmer). Temperature-dependent FTIR spectra were collected from a Varian Excalibur 3100 FTIR spectrometer by temperature-controlled IR cell.



Figure S1 UV-vis spectra of 0.6mM hemoglobin (black), 0.6mM hemoglobin-imidazole (red) and 0.6mM hemoglobin-CN (blue) in D2O

**Reference**

(1) Li, G. F.; Magana, D.; Dyer, R. B. *J. Phys. Chem. B* **2012**, *116*, 3467.

(2) Li, G. F.; Magana, D.; Dyer, R. B. *Nat. Commun.* **2014**, *5*, 3100.