**Supporting Information**

Energy Flow and Allostery 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.{Li, 2012 #36;Li, 2014 #37} 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



Figure 4 (A) TRIR spectral comparison at 2 ps of 0.6mM hemoglobin, 0.6mM hemoglobin-CN and 0.6mM hemoglobin-imidazole in D2O; (B) Normalized IR transient comparison at 1630 cm-1 of 0.6mM hemoglobin, 0.6mM hemoglobin-CN and 0.6mM hemoglobin-imidazole in D2O.



Figure 2 IR transient comparison of 2.5mM hemin (black) and 0.6mM methemoglobin (red) in D2O at 1630 cm-1 (A), 1467 cm-1 (B), 1425 cm-1 (C), 1600 cm-1 (D), 1407 cm-1 (E) and 1370 cm-1 (F)

For heating bands at 1425 and 1467 cm-1 in amide II range, heating signals become maximum at ~3 ps, and becomes equilibrium in 40-50 ps. In comparison with amide I heating at 1630 cm-1, amide II band heating at 1467 cm-1 shows a faster rise and fast equilibrium process. The similar results were observed from metalloporphyrin and albumin system. The dynamics difference between amide I and II is related to transient signals overlaps (positive and negative bands from heme and globin heating) in amide II range. After 40-50 ps, no any heme interference exists since heme relaxes to ground state evidenced by dynamics of heme at 1407 cm-1 (Figure 2E). Heme in hemoglobin relaxes to ground states at 4.6 ps, which is faster than hemin in water (7.6 ps). However, dynamics at 1370 cm-1 in Figure 2F clearly shows no dynamics difference between hemin and heme of hemoglobin. Therefore, 3 ps fast process at 1407 cm-1 is due to band overlaps of positive globin heating and negative heme/globin bands. Energy flows from hemin and hemoglobin to water are almost same in figure 2D.

Since metHb does not bind oxygen due to the oxidation state of the heme, we seek to additionally probe whether altering the heme spin state affects the transient response of the protein. To probe this effect, we performed two independent controls with exogenous ligands, cyanide and imidazole, respectively. Binding of imidazole and cyanide induce a redshift of the Soret band from 406nm to 412nm and 420nm, respectively (c.f. Fig. S1). In addition, the heme Q-bands exhibit a mild increase in absorption and a redshift of ~30 nm. These spectral changes are indicative of a change in spin state from high-spin to low-spin heme, which is expected for 6-coordinate heme complexes.{Verma, 1974 #34}

Figure 4A and 4B compare TRIR spectra and dynamics of hemoglobin, hemoglobin-CN and hemoglobin-imidazole in water. TRIR spectral red shift of hemoglobin-CN and hemoglobin-imidazole around 1570 cm-1 may be induced by environmental change of heme accompanying with binding cyanide and imidazole. But, no distinct TRIR spectral changes are observed, and their dynamics at 1630 cm-1 are almost same. These results indicate that external ligands binding on iron of heme don’t cause energy flow change in hemoglobin. Therefore, energy flow difference at 1630 cm-1 in Fig. 3B in the main text should be only explained by hydration change in hemoglobin. Hydration changes on R and T state of hemoglobin have been investigated by allosteric effectors and neutral solvents. Additional water molecules in oxygen states (R state) of hemoglobin were observed.{Colombo, 1999 #10;Salvay, 2003 #35;Colombo, 1996 #31}

**References**