Energy Flow and Allostery in Hemoglobin

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Supporting Information

ABSTRACT: By visible excitation of heme, energy flow in hemoglobin has been successfully observed from heme to globin by femtosecond time resolved infrared spectroscopy. Further, energy flow has been utilized to understand correlation between hydration and allosteric effect of hemoglobin. External ligands (cyanide and imidazole) binding on heme of hemoglobin don’t induce energy flow change evidenced by heating signals in amide I range. Further results show T state of hemoglobin induced by ethylene glycol shows fast energy flow than R state, which suggests hydration change in hemoglobin plays a significant role in allosteric effect rather than ligand binding by considering external ligands and allosteric effector binding results.

It is well known that protein hydration plays a significant role in protein dynamics and functions such as enzyme catalysis and allosteric effects,{Fenimore, 2002 #12}{Daniel, 2003 #1}{Kurkal, 2005 #2}{Frauenfelder, 2007 #3}{Colombo, 1992 #4} and hemoglobin (Hb) has been extensively investigated as model system for understanding dynamic, allosteric phenomena.{Colombo, 1992 #4}{Yonetani, 2008 #7}{Baldwin, 1979 #5}{Hundahl, 2003 #6}{Perutz, 1993 #8}{Takayanagi, 2014 #9} In transition between the oxygenated (relaxed/R) and deoxygenated (tense/T) conformations, several direct contacts between subunits are broken and exposed to solvent, which implies a difference in hydration between the two limiting conformations of Hb.{Yonetani, 2008 #7}{Hundahl, 2003 #6}{Perutz, 1993 #8}{Takayanagi, 2014 #9}{Haire, 1983 #11}{Perutz, 1979 #30} Indeed, prior work indicates that the fully oxygenated R-state of Hb is hydrated by as many as ~60 additional water molecules compared to the deoxygenated conformer, as well as an addtional ~10 water molecules in the transition state.{Colombo, 1992 #4}{Colombo, 1999 #10}{Salvay, 2003 #35}{Goldbeck, 2001 #40} With this in mind, we seek to probe how changes in hydration facilitate the transfer of the free energy of effector binding to the heme sites in Hb, thereby modulating its oxygen binding affinity.

Molecular dynamics (MD) simulations have already demonstrated existence of coupling between protein and water,{Nibali, 2014 #13;Shenogina, 2008 #14;Heyden, 2010 #15} which was suggested by THz, fluorescence and ultrafast optical Kerr effect studies.{Heyden, 2010 #15;Mazur, 2012 #17;Zhong, 2009 #20} MD simulation also predicted that low frequency modes are strongly coupled with water while intermediate and high frequency modes are decoupled with water.{Shenogina, 2008 #14} Neutron scattering and THz techniques indicated thermal coupling between protein and water is effective on picosecond timescale.{Paciaroni, 2008 #18;Paciaroni, 2008 #18;Lipps, 2012 #19} Since water on protein surface is main driving force for protein dynamics,{Combet, 2012 #22} water on protein surface has been well characterized by fluorescence, ultrafast optical Kerr effect and interfacial spectroscopy.{Mazur, 2012 #17;Zhong, 2009 #20;Engelhardt, 2014 #21;Yang, 2012 #23;Pal, 2002 #25} But not much research has focused on dynamics of the protein matrix. Although THz and far-infrared technique could provide low frequency information of protein matrix, it is hard to separate protein signal from low frequency of water since both of them have broad bands overlapped each other.{Lipps, 2012 #19}

By depositing a large excess of energy into the heme group of Hb, the heat dissipation of each limiting conformation can be directly examined via the vibrational bands of the amide backbone. Prior experiments using picosecond time-resolved infrared (TRIR) to study protein dynamics in carbonmonoxymyoglobin indicate that CO photolysis, and the subsequent energy deposition into the heme triggers an allosteric transition between the limiting conformers within 6-8 ps,{Causgrove, 1996 #47} and has been recently shown via femtosecond X-ray solution scattering measurements to induce a ballistic “proteinquake” in the initial stages of thermal equilibration.{Levantino, 2015 #49} These results have been further corroborated by our recent work, where we observed combined ballistic and diffusive heat transport in albumin complexes with Fe and Cu porphyrins using ultrafast TRIR spectroscopy. We also found that the rate of heat flow remains unchanged whether the heme chromophore is covalently or non-covalently bound to albumin. Additionally, enhanced ballistic heat transport was observed by subsequent binding of an allosteric effector, sodium myristate. {Li, 2014 #37}[ref JPCL]

In this Communication, we measure the differences in heat flow kinetics resulting from the allosteric R-T transition of methemoglobin (metHb) via dehydration with ethylene glycol. In order to compare the allosteric contributions from hydration and ligand binding, we also probe the heat flow dynamics upon addition of exogenous ligands such as imidazole and cyanide. The results below are then put into context with differences in hydration in both ferrous hemoglobin and methemoglobin.

The comparison between the transient and equilibrium heating response is given in Figure 1A, which shows the ultrafast TRIR (10 ps delay) and temperature-dependent FTIR (T-FTIR, 23.8oC-20.3oC) difference spectra of 0.6 mM metHb in D2O. To monitor the evolution of the transient heating response, and to characterize the heme and protein IR bands, respectively, figure 1B compares the evolution of the TRIR difference spectra of hemin and metHb at various pump delays. Both hemin and metHb exhibit ground state bleaches at 1407cm-1 and 1556 cm-1, which correspond to the symmetric and asymmetric carboxylate stretches of heme, respectively, accompanied by broad excited state heating band at 1383 cm-1.{Nonaka, 1990 #28} The ground state bleach at 1466 cm-1 is assigned to amide II, with its excited state heating band centered at ~1425 cm-1, overlapping with the heme bleach. The bleach at 1630 cm-1 is assigned to amide I, and we monitor the solvent response at 1600 cm-1. A broad positive heating band at 1665 cm-1 is seen in the T-FTIR spectrum, which is absent in the TRIR spectra. This band is likely due to conformational changes at long times, similar to what we have reported previously for heme-albumin complexes.{Li, 2014 #37} Additionally, Fig 1A shows that the heating signals of heme and amide II in the T-FTIR spectrum are redshifted by ~10 cm-1 as compared to the 10 ps TRIR spectrum. This redshift at early times is likely due to the competition between the overlapping amide II excited state band with the heme ground state bleach as these signals evolve.

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)

Figure 1 (A) comparison of differential FTIR spectra (23.8oC-20.3oC) and TRIR spectra (10 ps) of 0.6mM methemoglobin in D2O; (B) TRIR spectral comparison of 2.5mM hemin (black) and 0.6mM methemoglonbin (red) in D2O at different time scales. TRIR spectra in (B) are offset for clarity.

In addition to the above-mentioned characterization, we can observe the overall heat flow path via the TRIR spectra. At early times (3 ps), the heme signal contributes significantly to the transient IR response. Within 10 ps, the heme signal has decayed almost completely, while the protein bands increase in intensity between 10-20 ps. The presence of a substantial ground state bleach at ~1556 cm-1 at times >10 ps is likely due to the carboxylate groups of the protein (i.e., Asp/Glu residues). By 100 ps, most of the heat has flowed out of the protein and into the solvent. This is illustrated further by IR transient analysis, given below.

Figure 2 shows the transient heating response measured for the heme group (1407 cm-1), amide I band (1630 cm-1), and the solvent (1600 cm-1) for hemin and metHb in D2O. Further transient analysis of the amide II response and excited vibrational bands is given in the Supporting Information. Upon absorption of a 400 nm photon by the heme, ultrafast heating occurs,{Consani, 2014 #50} followed by rapid heat transfer to the protein structure and solvent (4.6 ps for metHb, 7.6 ps for hemin). Ballistic amide I heating follows in metHb with a weighted average time constant of ~4 ps (~400 fs and 3.6 ps components) followed by decay of the heating signal with a time constant of ~43 ps, which does not cool to equilibrium within the 100 ps time window. Ballistic heat transport is evidenced in the 1600 cm-1 transients of both hemin and metHb, which decay biexponentially with weighted average time constants of 3.7 ps (360 fs and 8 ps components), and 8.5 ps (200 fs and 11 ps decay components), respectively.

It is well established that the transition between the oxy (R) and deoxy (T) states of Hb can be induced by modulating the degree of protein hydration via addition of ethylene glycol (EG), sucrose, stachyose, or glycine.{Colombo, 1992 #4;Hundahl, 2003 #6;Haire, 1983 #11;Colombo, 1996 #31} Since the EG cosolvent interacts weakly with Hb, modulation of oxygen binding affinity can only be observed at high EG concentrations (0.2–0.4 mole fraction, 47–70% EG w/w). Within this range, the oxygen binding affinity of Hb has been shown to decrease.{Haire, 1983 #11} In the TRIR experiments discussed below, we dehydrate the protein with the lowest mole fraction of 0.2, which effectively induces the structural transition from the R-state to the T-state, while maintaining stability of the protein.

Figure 3A shows the TRIR spectra at 2 ps of metHb in D2O and in 0.2 mole fraction EG/D2O. Since TRIR spectra change around 1630 cm-1 is only related to amide I heating, hydration effect on energy flow in hemoglobin should be disclosed by amide I. It is interesting to notice that heating band of amide I at EG/water (0.2 mole fraction) becomes narrow and is shifted to low wavenumber, which suggests structure of hemoglobin’ T state is more order and compact than its R state. Therefore, energy flow in T state is expected to be faster than R state. Figure 3B clearly shows equilibrium of energy flow in T state is 19.4 ps which is faster than R state (37.3 ps). Energy flow rate from heme to globin is extremely fast (< 1 ps). From Fig.3B, we can notice the heating curves features immediately after exciting heme. In our previous energy flow work on albumin, we discussed low frequency mode coupling between heme and protein matrix is main channel of energy flow no matter if covalent bond exists between chromophore and protein matrix. MD simulation has also demonstrated only low frequency mode of protein could strongly couple with interfacial water while high frequency mode weakly couples with water on protein.{Shenogina, 2008 #14} Unfortunately, we can’t detect energy flow rate difference due to low frequency mode coupling at different hydrations since time scale of this coupling is extremely fast. Leitner’ group predicted low frequency mode damping rates of chromophore is around subpicosecond level.{Leitner, 2012 #33}

It has been shown that both oxyHb and deoxyHb have characteristic features in their circular dichroism (CD) spectra around 280 nm, which characterize the R-to-T transition. To definitively ascribe any alteration in heat flow dynamics to an R-to-T transition, we have measured CD spectra for metHb. MetHb has been shown to undergo a less pronounced R-to- T transition in its CD spectra via addition of the allosteric effector inositol hexaphosphate,{Perutz, 1974 #59} and we have obtained similar results using EG (c.f. Supporting Information).

Figure 3 (A) TRIR spectral comparison at 2 ps of 0.6mM hemoglobin in D2O and ethylene glycol/D2O (0.2 mole fraction); (B) IR transient comparison at 1630 cm-1 of 0.6 mM methemoglobin in D2O and ethylene glycol/D2O (0.2 mole fraction) [need to add 1600 cm-1 tranients to B]

Numerous results indicate that water plays a critical role in protein structure and function, whether it is associated on the protein surface,{Fenimore, 2002 #12}{Combet, 2012 #22}, within a binding pocket or as an external ligand.{Esquerra, 2010 #51} We hypothesize that the enhanced ballistic heat flow in metHb in the presence of EG is likely due to a net dehydration of the binding pocket due to the dehydrating effects of EG, which would result in the heme group adopting a domed conformation, in closer proximity to the proximal histidine when external ligands are absent.{Park, 2006 #52} Our previous results on heme-albumin complexes indicate that excess thermal energy is efficiently dissipated through the protein and out to the bulk solvent via through-space anharmonic coupling of low-frequency heme modes (i.e. doming and ruffling modes 80 cm-1) to the low-frequency protein structural modes.[ref JPCL]{Kubo, 2008 #58}{Deng, 2002 #56}{Shi, 2003 #57} Additonally, due to the net dehydration of the protein surface in the T-state, the protein structure becomes more rigid due to surface dehydration, which should result in more efficient coupling of heme low-frequency modes to the low-frequency protein structural vibrations.

In ferrous hemoglobin, is well-known that the protein surface in the R-state (oxy) is hydrated by ~60 additional water molecules as compared to the T-state (deoxy), regardless of the presence of allosteric effectors.{Colombo, 1992 #4}{Colombo, 1999 #10}{Salvay, 2003 #35} Additionally, nanosecond protein relaxation dynamics of HbCO following CO photolysis indicate that the transition state from R-to-T is hydrated by an additional ~10 water molecules prior to the net dehydration of the surface as the T-state equilibrates. {Goldbeck, 2001 #40} In contrast to the protein surface, the hydration state of the heme pocket shows the opposite trend, in that the heme pockets are not hydrated in oxyhemoglobin, due to bound oxygen excluding water from the binding pocket, whereas the **-subunits of deoxyhemoglobin contain water.{Esquerra, 2010 #51}{Park, 2006 #52} Thus, for ferrous hemoglobin, there is a net hydration of the heme pockets in the R-T allosteric transition. However, in metHb, both the *-* and **-subunits are hydrated in the R-state (since metHb does not bind O2),{Deatherage, 1976 #55} and assuming similar T-state hydration to deoxyhemoglobin, the R-T transition in metHb should yield a net dehydration of the binding pockets and consequently, more efficient coupling of the low-frequency heme modes through the protein and out to the solvent.

In summary, anisotropic energy flow in hemoglobin has been successfully demonstrated by comparing with TRIR results of hemin and temperature-dependent FTIR of hemoglobin. Heating bands of globin in amide I and amide II range were observed by fs time resolved infrared spectroscopy for the first time. By cosolvent of ethylene glycol and water, energy flow in R and T states of hemoglobin could be selected to be studied. Heating band in amide I is shifted to low frequency and becomes narrow for T state of hemoglobin. Energy flow equilibrium time in T states is faster than R state, which is due to more interfacial water on T states by considering external ligands binding results. Current results suggest hydration on protein surface and interface plays key role on allosteric effect.

ASSOCIATED CONTENT

Supporting Information

The materials and sample preparation, description fs laser system, details of the UV-Vis and T-FTIR measurements, and related spectra are given as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes  
The authors declare no competing financial interest.

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REFERENCES

(1)Fenimore, P. W.; Frauenfelder, H.; McMahon, B. H.; Parak, F. G. Proc. Natl. Acad. Sci. U.S.A. 2002, 99, 16047.

(2)Daniel, R. M.; Dunn, R. V.; Finney, J. L.; Smith, J. C. Annu. Rev. Biophys. Biomol. Struct. 2003, 32, 69.

(3)Kurkal, V.; Daniel, R. M.; Finney, J. L.; Tehei, M.; Dunn, R. V.; Smith, J. C. Biophys. J. 2005, 89, 1282.

(4)Frauenfelder, H.; Fenimore, P. W.; Young, R. D. Iubmb Life 2007, 59, 506.

(5)Colombo, M. F.; Rau, D. C.; Parsegian, V. A. Science 1992, 256, 655.

(6)Yonetani, T.; Laberge, M. Biochim. Biophys. Acta 2008, 1784, 1146.

(7)Baldwin, J.; Chothia, C. J. Mol. Biol. 1979, 129, 175.

(8)Hundahl, C.; Fago, A.; Malte, H.; Weber, R. E. J. Biol. Chem. 2003, 278, 42769.

(9)Perutz, M. F.; Fermi, G.; Poyart, C.; Pagnier, J.; Kister, J. J. Mol. Biol. 1993, 233, 536.

(10)Takayanagi, M.; Kurisaki, I.; Nagaoka, M. Sci. Rep. 2014, 4.

(11)Haire, R. N.; Hedlund, B. E. Biochemistry-Us 1983, 22, 327.

(12)Perutz, M. F. Annu. Rev. Biochem. 1979, 48, 327.

(13)Colombo, M. F.; Seixas, F. A. V. Biochemistry-Us 1999, 38, 11741.

(14)Salvay, A. G.; Grigera, J. R.; Colombo, M. F. Biophys. J. 2003, 84, 564.

(15)Nibali, V. C.; D'Angelo, G.; Paciaroni, A.; Tobias, D. J.; Tarek, M. J. Phys. Chem. Lett. 2014, 5, 1181.

(16)Shenogina, N.; Keblinski, P.; Garde, S. J. Chem. Phys. 2008, 129.

(17)Heyden, M.; Havenith, M. Methods 2010, 52, 74.

(18)Mazur, K.; Heisler, I. A.; Meech, S. R. J. Phys. Chem. A 2012, 116, 2678.

(19)Zhong, D. Adv. Chem. Phys. 2009, 143, 83.

(20)Paciaroni, A.; Orecchini, A.; Cornicchi, E.; Marconi, M.; Petrillo, C.; Haertlein, M.; Moulin, M.; Sacchetti, F. Philos. Mag. 2008, 88, 4071.

(21)Lipps, F.; Levy, S.; Markelz, A. G. Phys. Chem. Chem. Phys. 2012, 14, 6375.

(22)Combet, S.; Zanotti, J. Phys. Chem. Chem. Phys. 2012, 14, 4927.

(23)Engelhardt, K.; Peukert, W.; Braunschweig, B. Curr. Opin. Colloid Interface Sci. 2014, 19, 207.

(24)Yang, J.; Zhang, L. Y.; Wang, L. J.; Zhong, D. P. J. Am. Chem. Soc. 2012, 134, 16460.

(25)Pal, S. K.; Peon, J.; Zewail, A. H. Proc. Natl. Acad. Sci. U. S. A. 2002, 99, 1763.

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

(27)Nonaka, Y.; Lu, D. S.; Dwivedi, A.; Strommen, D. P.; Nakamoto, K. Biopolymers 1990, 29, 999.

(28)Castellanos, M. M.; Colina, C. M. J. Phys. Chem. B 2013, 117, 11895.

(29)Colombo, M. F.; BonillaRodriguez, G. O. J. Biol. Chem. 1996, 271, 4895.

(30)Hilinski, E. F.; Straub, K. D.; Rentzepis, P. M. Chem. Phys. Lett. 1984, 111, 333.

(31)Leitner, D. M. Chem. Phys. Lett. 2012, 530, 102.

(32)Verma, A. L.; Bernstein, H. J. J. Raman Spectrosc. 1974, 2, 163.

(33)Gnanasekaran, R.; Xu, R.; Leitner, D. M. J. Phys. Chem. B 2010, 114, 16989.

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