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 function such as enzyme catalysis and allosteric effects.[1-5](#_ENREF_1) Since hemoglobin can transport oxygen in red blood cells, it has been extensively investigated as model system for understanding protein dynamics and allosteric effect.[5-10](#_ENREF_5) Most researches focused on R and T transition of hemoglobin evidenced by binding affinity change of oxygen.[6](#_ENREF_6),[8-12](#_ENREF_8) Further results disclosed that hydration at R state of hemoglobin is higher (~60 extra water molecules) than T states no matter which allosteric effectors are present in hemoglobin solution.[5](#_ENREF_5),[13](#_ENREF_13),[14](#_ENREF_14) This result brings up an interesting question, the mechanism of allosteric effect. What is the fundamental factor that causes R and T transition of hemoglobin? Recently, observation of non-site-specific allosteric effect in hemoglobin has challenged traditional understanding of allosteric effect.[10](#_ENREF_10)

In order to clarify above issue, correlation of water and protein’ allosteric effect should be fully understood. Molecular dynamics (MD) simulations have already demonstrated existence of coupling between protein and water,[15-17](#_ENREF_15) which was suggested by THz, fluorescence and ultrafast optical Kerr effect studies.[17-19](#_ENREF_17) MD simulation also predicted that low frequency modes are strongly coupled with water while intermediate and high frequency modes are decoupled with water.[16](#_ENREF_16) Neutron scattering and THz techniques indicated thermal coupling between protein and water is effective on picosecond timescale.[20](#_ENREF_20),[21](#_ENREF_21) Since water on protein surface is main driving force for protein dynamics,[22](#_ENREF_22) water on protein surface has been well characterized by fluorescence, ultrafast optical Kerr effect and interfacial spectroscopy.[18](#_ENREF_18),[19](#_ENREF_19),[23-25](#_ENREF_23) 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.[21](#_ENREF_21)

Recently, we have successfully observed energy flow in albumin by probing protein heating band using femtosecond time-resolved infrared (TRIR) technique.[26](#_ENREF_26) Once allosteric effector, myristate acid, binds with albumin, energy flow becomes faster, which could be used to investigate correlation of water and protein coupling. In current work, energy flow in hemoglobin will be firstly demonstrated by probing amide I and II heating bands. Further, energy flows at R and T state of hemoglobin are probed by the dehydration effect induced by ethylene glycol. In order to clarify allosteric effect contribution from hydration and ligand binding, energy flow will be discussed when hemoglobin covalently binds with external ligands such as imidazole and cyanide.

Figure 1A shows a comparison of the ps TRIR and differential FTIR (23.8oC-20.3oC) of 0.6 mM hemoglobin in D2O. Two negative bands at 1407 cm-1 and 1556 cm-1 are attributed to symmetric and asymmetric COO- of heme group in hemoglobin, respectively.[27](#_ENREF_27) The bands at 1466 cm-1 and 1630 cm-1 are assigned to hemoglobin heating in amide II and I range. The broad protein heating band at 1665 cm-1 in FTIR is absent in TRIR spectra, which is similar to heating profiles of albumin published previously.[26](#_ENREF_26) This suggests energy flow in hemoglobin is anisotropic at current time scales. It is unclear for assignments of two positive bands at 1383 cm-1 and 1425 cm-1 in TRIR. Also, we noticed that these two bands are shifted to 1393 cm-1 and 1435 cm-1, respectively. This difference may be related to evolution difference of transient bands from positive and negative bands of heme or globin. The comparison of transient evolution from hemoglobin and hemin is exhibited in figure 1B. Any difference in Fig. 1B is related to globin and assigned to protein matrix heating. It is clear that the bands at 1425, 1466, 1550 and 1630 cm-1 are due to globin heating, and the band at 1383cm-1 is assigned to excited states of heme group in hemoglobin. It is interesting to observe that globin bands become intense around 10ps, and its intensity decreases after 10 ps.

Figure 2(A)-(C) compares the transient difference at globin heating range (1630, 1467 and 1425 cm-1) between hemin and hemoglobin. For hemin, only water heating profiles were observed at 1630 and 1467 cm-1 while hemoglobin exhibits globin heating and energy flow equilibrium process in globin. Once heme group is excited by 400 nm, globin heating immediately was observed (less 1 ps), and heating signal reaches maximum around 10ps at 1630 cm-1. Energy flow process becomes equilibrium after ~60 ps, and heating signals last at long time scales (ns). Our previous T-jump IR results on albumin show protein experiences structural change at ns level,[28](#_ENREF_28) which matches theoretical estimation (50-100ns) of protein.[26](#_ENREF_26) 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.

Figure 2 IR transient comparison of 2.5mM hemin (black) and 0.6mM hemoglobin (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)

It was well established that R and T states of hemoglobin could be modulated by degree of hydration in protein induced by ethylene glycol (EG), sucrose, stachyose and glycine.[5](#_ENREF_5),[8](#_ENREF_8),[11](#_ENREF_11),[29](#_ENREF_29) In current work, we controlled hydration in hemoglobin by changing concentration of ethylene glycol in water. Since cosolvent has week interaction with hemoglobin, change of oxygen binding is only observed under high concentration of EG.[11](#_ENREF_11) It was reported that oxygen binding affinity is only decreased in the range of EG mole fraction up to 0.2. After 0.2 mole fraction (0.2~0.4), oxygen binding affinity will be increased.[11](#_ENREF_11) Therefore, 0.2 mole fraction of EG is critical point for T state of hemoglobin. In following discussion, we will compare energy flow difference of R and T states of hemoglobin in water and EG/water (0.2 mole fraction).

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

Figure 3A shows TRIR spectra of hemoglobin in water and EG/water (~0.2 mole fraction) at 2.0 ps by exciting heme group. Since the broad band at 1565 cm-1 includes heme and partial amide II information, it is not easily to separate them out. It is well known that lifetime of metalloporphyrin is strongly dependent of environments change such as solvents’ hydrophilic and hydrophobic properties.[30](#_ENREF_30) Therefore, the difference of broad at 1565 cm-1 may be related to dynamics change of heme group in hemoglobin at different hydration environments. 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.[16](#_ENREF_16) 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.[31](#_ENREF_31)

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 hemoglobin in D2O and ethylene glycol/D2O (0.2 mole fraction)

In order to confirm if above energy flow difference is related to binding and unbinding of oxygen on heme group, cyanide and imidazole are used as external ligands of hemoglobin to check this point. Since cyanide and imidazole are small ligands, hydration in hemoglobin is assumed to be same. Therefore, we could check ligands binding effect on heme group by these external ligands. Binding of imidazole and cyanide induce red shift of Soret band from 406nm to 412nm and 420nm, respectively (see figure S1), which is due to binding of hydrophobic group on heme. At the same time, Q bands distinctly changes after external ligands binding, which are indicator of iron transition change from high spin to low spin states.[32](#_ENREF_32)

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 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.[13](#_ENREF_13),[14](#_ENREF_14),[29](#_ENREF_29)

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

In order to get clear correlation between energy flow and hydration in protein, we need understand types of water in protein. It was reported that surrounding solvent in protein could be defined as nonslaved and slaved type.[1](#_ENREF_1) Only small portion of solvent in protein could slave dynamics of protein. Therefore, it is not correct to simply relate hydration in protein with allosteric effect or energy flow. It has been reported that interfacial water is main driving force for protein dynamics.[22](#_ENREF_22) So, it is more reasonable to build correlation between interfacial water and allosteric effect. MD simulation suggested that interfacial waters on hemoglobin could enhance vibrational energy transport across the interface by 10%.[33](#_ENREF_33) In compare with R state of hemoglobin, T state has less hydration in protein. But, more interfacial water should be present in T state of hemoglobin since ethylene glycol takes more bulk water (unslaved) from protein.

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

Chemicals and sample preparation of hemin, hemoglobin, hemoglobin-CN and hemoglobin-imidazole; fs laser system, UV-vis, FTIR spectrometer; UV-vis spectra of hemoglobin with cyanide and imidazole. 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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