Femtosecond Time-Resolved Absorption of Oxyhaemoglobin Photolysis in Living Erythrocytes *

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By using the pump–probe technique, the femtosecond (fs) time-resolved absorption dynamics of oxyhaemoglobin (oxy Hb) in living erythrocytes was studied for the first time to our knowledge. The changing course of erythrocyte absorption at 400 nm was found to be composed of three exponential decay components with the time constants of 200 ± 28 fs, 3.60 ± 0.14 ps and 215 ± 21 ps respectively, which are consistent with those of oxy Hb water solution measured in the same way, indicating that the fast process (less than 1 ns) of photo-induced response in erythrocytes was as the same as that in oxy Hb aqueous solution. These three decay components were assigned to the transitions from excited states of unligated haemoglobin (Hb^*_I, Hb^*_{II}) and fast geminate recombination of O_2 and Hb, respectively.

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Haemoglobin (Hb) is the primary oxygen-transport protein in all vertebrate organisms. In an environment of high oxygen concentration, the oxygen is bound to Hb in a ligand form becoming oxyhaemoglobin (oxy Hb) with the structure known as the R state, and released from oxy Hb in an environment of low oxygen concentration becoming deoxyhaemoglobin (deoxy Hb) with the structure of the T state. Due to the unique role of Hb played in physiology, as well as the corresponding relationship between the biological function and molecular structure, the properties of haemoglobin–ligand binding have been extensively studied.[1−3] During the past two decades, obvious progress on the mechanism research of association and dissociation between Hb and ligand molecules had been made. The time scale of photolysis kinetics has been pressed back from the microsecond (μs), nanosecond (ns), and picosecond (ps), to femtosecond (fs).[4−6] From previous reports, the dynamics of oxy Hb disassociation and recombination can be concluded as several steps, such as the excited states of Hb, the fast and slow geminate recombination of O_2 and Hb, the protein conformation change and the combination of Hb and exogenous O_2. For the slow course (ns–μs), the understanding from different reports were comparable.[5−7] However, for the fast course (ps) concerned fast geminate recombination, there are different points of view. Petrich *et al.*[4] suggested that 2.5 ps is the time constant for the fast course of O_2 rebinding with Hb^*_{II} (excited state of Hb), while other researchers reported that the fast geminate recombination takes 100–200 ps.[5,7] Different understandings still exist for this fast kinetics. On the other hand, though many studies have been carried out in this field, only a few works experimentally explored the photolysis dynamics of oxy Hb in living erythrocytes. In particular, so far there has been no report on the fast course (ps) of photo-induced response in living erythrocytes. In erythrocytes, the oxy Hb molecules are mixed with many other biomolecules and enveloped by erythrocyte's membrane. The concentration of oxy Hb in erythrocytes is as high as 10 mmol/L. Under such a condensed condition and in the complex environment in erythrocytes, it has not yet been answered whether the conformation of oxy Hb molecules is affected so that the behavior of oxy Hb photolysis is changed compared to that of its aqueous solution.

In this Letter, by using a short-pulsed laser and one-colour pump–probe technique, we study the femtosecond time-resolved dynamics of photolysis of oxy Hb in aqueous solution from 0.1 ps to 100 ps, which is comparable with that in living erythrocytes in parallel to explore the photo-induced dynamics of oxy Hb in physiological condition.

Fresh human blood was obtained by venipuncture from healthy volunteers and kept in glass tubes containing heparin sodium as an anti-coagulant. The erythrocytes were collected from the whole blood by centrifuging, and then washed with phosphate buffered saline (PBS, PH 7.4) three times. The erythrocyte suspension in PBS was prepared in different haematocrits for measurements.

Human haemoglobin was made from the haemolysis of fresh erythrocytes, by mixing erythrocytes with purified water (1:3) and then removing the ghost by centrifuging. The stock solution of the haemoglobin was 2.3 mM. The haemoglobin is in the oxy form (oxy Hb) in air. To prepare deoxyhaemoglobin (deoxy Hb), a small amount of sodium dithionite ($Na_2S_2O_4$,

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2.5 mg/mL) was added to the haemoglobin aqueous solution.

For confirming the different state (oxy or deoxy form) of Hb in solution as well as in erythrocytes, the absorption spectra were measured on a HITACHI U-500 UV-spectrometer in the 350–700 nm range.

The one-colour femtosecond pump–probe experiment was conducted with a home-built system.[8] The ultrafast pulses from a Ti:sapphire laser (Tsunami, Spectra Physics) served as the seed for a Ti:sapphire regenerative amplifier (Spitfire, Spectra Physics) which was pumped with the ND:YLF laser. The output pulses with the wavelength of 800 nm and 10 nm bandwidth were of 140 fs duration with a repetition rate of 1 kHz. By using a frequency doubling crystal (BBO crystal), the 800 nm laser pulses were turned to 400 nm in wavelength. In experiments, this 400 nm laser beam (duration is also around 140 fs) was divided into pump beam and probe beam with a beam splitter and the ratio of pump-to-probe power was set to 5:1. The pump beam passed through an optical delay line (ODL) and overlapped with the probe beam at the centre of the sample cell (1 mm in depth). The time delay between the probe and pump beams was controlled with the stepping motor in ODL instructed by a computer. A photodiode behind the sample cell was used to detect the probe beam, and with the help of a lock-in amplifier (SR830, Stanford Research System) the signal was fed to a computer for analysis. The pump power used in our measurement was less than 1 μJ/pulse with a beam diameter of about 50 μm in the beam overlap region.[9] All the measurements in this work were carried out at room temperature.

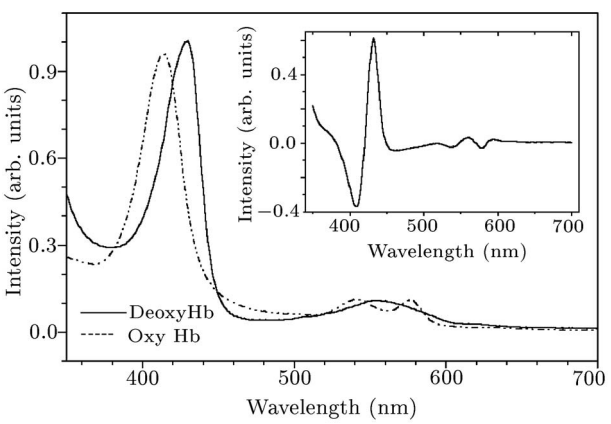


Fig. 1. Absorption spectra of deoxy Hb and oxy Hb with the concentration of 115 μM in purified water. The inset shows difference spectra of absorption between deoxy Hb and oxy Hb.

The absorption spectra of oxyhaemoglobin and deoxyhaemoglobin and their difference spectrum are shown in Fig. 1. The 400-nm wavelength locates in the main absorption band (Soret band) of oxy Hb, and also locates near the peak of the difference spectrum. Thus, the laser pulse with the wavelength of 400 nm was selected for both pump and probe beam in photolysis experiments.

For studying the fast course of oxy Hb photolysis in aqueous solution, the absorption change at 400 nm in 16 ps scale was detected first; the result is shown in Fig. 2. With the numerical fitting, this process is found to be composed of three exponential decay components with time constants of about 230 fs, 3.5 ps, and another slow one ($>$ 100 ps). No doubt the 230-fs component is relates to the transition of excited state (Hb_I^*) of Hb, and the slow component ($>$ 100 ps) might be involved in O_2 recombination. The interesting point is how to realize the component of 3.5 ps, which is a matter of debate.[4,5,7] Is it the event of oxygen-related combination or just the behaviour of the excited state of Hb? To explore the puzzle, the same fast course of deoxy Hb in aqueous solution was comparatively measured, as shown in Fig. 3. The typical feature is that it did not have the slow component ($>$ 100 ps) at all, since the probing signals have already decreased back to the baseline through a short decay course. This short decay course contains two components with the time constants of about 200 fs and 3.6 ps, respectively. Comparing the characteristic of photo-induced response between the oxy Hb and dexoxy Hb, the slow component with a long time constant in oxy Hb photolysis can be attributed to oxygen recombination. In the case of deoxy Hb, there were no oxygen molecules in the system, and the two components should represent the decay concerning the two excited states of deoxy Hb. It is known that there are two excited states for Hb, assigned as Hb_I^* and Hb_{II}^*.[4] In Fig. 3, the two components (200 fs and 3.6 ps) most probably represent the transition courses of haemoglobin molecules from excited states (Hb_I^* and Hb_{II}^*) to ground state respectively. In the case of oxy Hb, there were two fast components and one slow component in the whole process. May these two fast components come from exactly the same transition such as that of deoxy Hb? To verify this point, several measurements were repeatedly performed for the samples of both oxy Hb and deoxy Hb in aqueous solution, and the average time constants and corresponding standard derivations were calculated. They are 233 ± 81 fs and 3.59 ± 0.28 ps for oxy Hb, and 190 ± 43 fs and 3.60 ± 0.45 ps for deoxy Hb. To check the significance of the difference on time constants between the oxy Hb and deoxy Hb, the statistical analysis (t-test) was applied. The results of calculation show that the two p-values of these two components between the oxy Hb and deoxy Hb are both higher than 0.05, reflecting that there is no obvious difference statistically between the oxy Hb and deoxy Hb for this fast decay course. Petrich *et al.*[4] have experimentally demonstrated that the oxy Hb molecules were excited to unligated excited states Hb_I^* and Hb_{II}^* first, and these two excited states were involved in two decay components with the time constants of 300 fs and 2.5 ps. They assigned the first component (300 fs) to the transiting from Hb_I^* to the

ground state of deoxy Hb molecules, and the second component (2.5 ps) to the transition from Hb^*_{II} to the ground state of oxy Hb molecules combining the O_2 in the meantime. In our measurements, we also found two fast components with the time constants of 233 fs and 3.6 ps in the photo-induced course of oxy Hb, similar to the above reference data. The difference in absolute value between Petrich's data and the time constants we measured may be due to different measuring systems. However, it is demonstrated here that these two components of oxy Hb are almost the same as that of unligated deoxy Hb, and oxy Hb had another slow component (> 100 ps) in the process which Hb did not have. Based on this observation, the following assignment may be more reasonable. When the oxy Hb molecules were excited, they would be located at the Hb^*_I and Hb^*_{II} states accompanying the O_2 disassociation, and then transited back to the ground state of unligated deoxy Hb with time constants of about 233 fs and 3.6 ps respectively. These unligated deoxy Hb molecules in the ground state were combined with oxygen molecules recovering to the oxy Hb molecules (ground state) through the slow component course (> 100 ps).

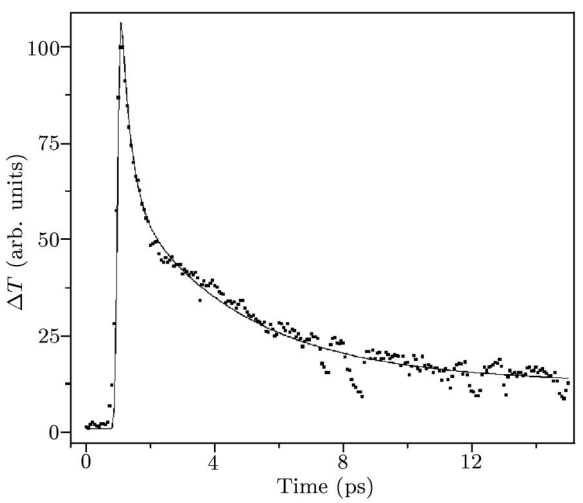


Fig. 2. Time-resolved absorption change of oxy Hb (150 μM) in PBS at 400 nm with one colour pump–probe technique. The dots are the experimental data measured. The solid line is the fitting.

To determine the time constant of the slow component in oxy Hb photolysis, the range of detection was extended to 100 ps. The result is shown in Fig. 4. The whole process of oxy Hb photolysis in this time scale contains three components, corresponding to the time constants 233 ± 81 fs, 3.59 ± 0.28 ps and 230 ± 26 ps respectively. The slow component (230 ps) can be attributed to the fast geminate recombination of O_2 and Hb. Therefore, the whole process includes the photophysics course (excited states) as well as the photochemistry course (ligand recombination). In Fig. 4, it is noticed that the signal decay does not reach the baseline, implying that the photolysis contains other even slower components. It is well known that, upon the photoinduced ligand dissociation, the haem relaxes from a planar six-coordinate low-spin haem to a five-coordinate high spin deoxy haem in the time scale less than 1 ns.[10] Meanwhile, the dissociated O_2 molecules are still in the haem pocket; some of them can be recombined with the deoxy haem turning to the oxy Hb with the so-called fast geminate recombination process, the others diffuse out of the haem pocket entering the region of bulk protein in the haemoglobin molecules. When these O_2 (in bilk protein) move back into the haem pocket, they can still combine themselves with deoxy haem, which is defined as slow geminate recombination, possessing the time scale of about 100 ns.[10] For fast geminate recombination, the time constants of 100–200 ps and 200 ± 70 ps were reported previously.[7,11] Considering the system difference, our data (230 ps) are in good agreement with the previous results.

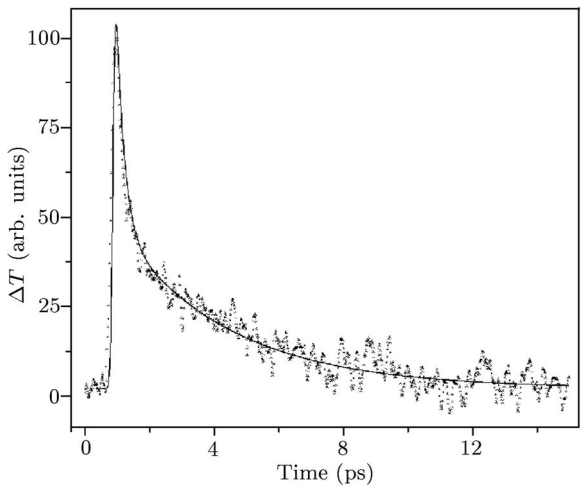


Fig. 3. Time-resolved absorption change of deoxy Hb (130 μM) in PBS at 400 nm with one-colour pump–probe technique. The dots are the experimental data measured. The solid line is the fitting.

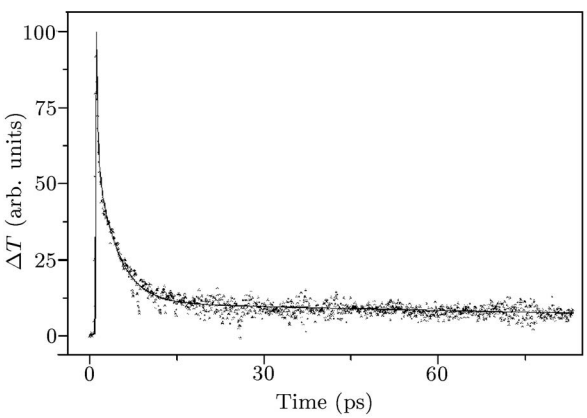


Fig. 4. Time-resolved absorption change of oxy Hb (150 μM) in PBS at 400 nm. The dots are the experimental data measured. The solid line is the fitting.

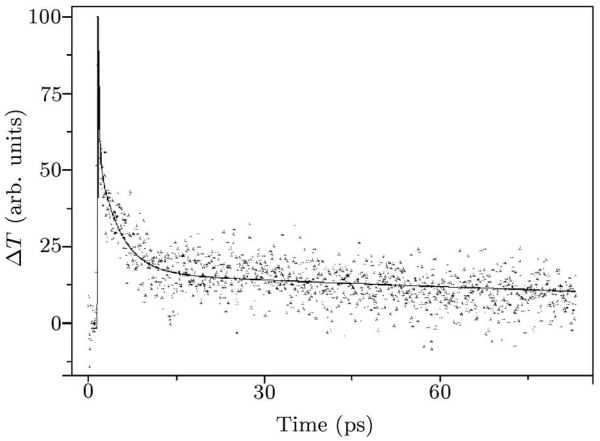


Fig. 5. Time-resolved absorption change of oxy erythrocytes in PBS (1.5% haematocrit) at 400 nm with one-colour pump–probe technique. The dots are the experimental data measured. The solid line is the fitting.

When the behaviour of oxy Hb photolysis in solution was clearly understood, the photolysis of oxy Hb in living erythrocytes was explored further. In the measurements of oxy Hb photolysis in aqueous solution, the concentration of haemoglobin was generally selected around the level of 100 μM.[4] However, the concentration of haemoglobin in living erythrocytes is as high as 10 mM,[6] and the scattering of erythrocytes causes much more noise in measurements. Thus, the selection of the sample haematocrit of erythrocytes is very important. By varying the haematocrit of erythrocytes in PBS, about 1% haematocrit was found to be suitable for photolysis measurements. Figure 5 shows the typical photolysis process of erythrocytes suspension with 1.5% haematocrit in air. Under the air condition, the haemoglobin molecules in erythrocytes are all in the oxy Hb state. The process of erythrocyte's photolysis also contains three components. They are 200 ± 28 fs, 3.60 ± 0.14 ps and 215 ± 21 ps respectively, and they are listed in Table 1 for comparison with oxy Hb aqueous solution. It seems to be obvious that these three components of photolysis in erythrocyte suspension are the same as that in oxy Hb aqueous solution. So far, only a few works have been reported to detect the slow course (100 ns lever) of photolysis in erythrocyte suspension[6] and the time constants were found to be markedly different from that of Oxy Hb in aqueous solution. The cell membrane of erythrocytes was suggested to be the main factor affecting oxygen recombination. Here the fast course (0.1–100 ps) of oxy Hb photolysis in living erythrocytes was studied for the first time. It is found that the three components of the course all are not different statistically compared to that of oxy Hb in aqueous solution, reflecting that in a 100-ps scale, the photolysis processes of oxy Hb in aqueous solution or in erythrocytes are in fact the same. The fast course (100 ps) is the indicator of excited states of Hb as well as the fast geminate recombination. Therefore, this course should be sensitive to the conformation change of oxy Hb. In erythrocytes the concentration of oxy Hb is as high as 10 mmol/L. The oxy Hb also mixed with many other bio-molecules in erythrocytes also, such as vitamin, reduced pyridine nucleotides (NADH) and glutathione (GSH) with the high concentration of 2.3 mmol/L. The result of Fig. 5 shows that the fast course of photo-induced dynamics in erythrocytes was unchanged compared to that in oxy Hb aqueous solution, implying that the relative condensed environment does not affect the conformation of oxy Hb in erythrocytes. The membrane of erythrocytes was reported to affect the slow course (100 ns level) in erythrocytes,[6] however it did not affect the fast course here. The behaviour of the fast course is only concerned with molecular conformation (excited states and haem pocket), regardless of the far-away surrounding such as membrane of erythrocyte.[12]

Table 1. Time constants of different components measured in photolysis courses (time constant τ).

Samples	τ_1 (ps)	τ_2 (ps)	τ_3 (ps)
Oxy Hb	233 ± 81	3.59 ± 0.28	230 ± 26
Deoxy Hb	190 ± 43	3.60 ± 0.45	
Oxy erythrocytes	200 ± 28	3.60 ± 0.14	215 ± 21

The fast course of photo-induced dynamics in erythrocytes suspension is concluded in three components. The two components (200 fs and 3.6 ps) belong to the transitions from the excited state to ground state. The third component (215 ps) is involved in rebinding of oxygen molecules, called the fast geminate recombination.

References

[1] Koshland D E, Nemethy G and Filmer D 1966 *Biochem.* **5** 365
[2] Hayashi A, Suzuki T and Shin M 1973 *Biochem. Biophys. Acta* **310** 309
[3] Demma L S and Salhany J M 1979 *J. Biol. Chem.* **254** 4532
[4] Petrich J W, Poyart C and Martin L J 1988 *Biochemistry* **27** 4049
[5] Ghelichkhani E, Goldbeck R A, Lewis J W and Kliger D S 1996 *Biophys. J.* **71** 1596
[6] Hoshino M, Sonoki H, Suzuki H, Adachi H, Miyazaki Y and Yamanaka K 2001 *J. Phys. Chem.* B **105** 10976
[7] Friedman J M, Scott T W, Fisanick G J, Simon S R, Findesn E W, Ondrias M R and Macdonald V W 1985 *Science.* **229** 187
[8] Guo L J, Liu Y, Yang Y, Mi J, Xu C, Xu C and Qian S 2002 *FEBS Lett.* **511** 69
[9] Guo L, Ma G, Zou Y, Xu C, Liu Y, Mi J, Qian S and Liu J 2001 *Chin. Phys. Lett.* **18** 522
[10] Friedman J M 1985 *Science.* **228** 1273
[11] Chernoff D A, Hchstrasser R M and Steele A W 1980 *Proc. Natl. Acad. Sci.* U.S.A. **77** 5606
[12] Martin J L 1983 *Proc. Natl. Acad. Sci.* U.S.A. **80** 173