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# Laser-induced time-resolved photoacoustic calorimetry study on photo-dissociation of human and bovine oxyhemoglobin

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**Abstract**

The dynamics of the enthalpy and volume changes related to the photo-dissociation of oxygen from human and bovine oxyhemoglobin are investigated by nanosecond time-resolved photoacoustic calorimetry (PAC). The values of enthalpy and volume change associated with the above process are $\Delta H = 37.8 \pm 3$ kcal/mol, $\Delta V = 5.0 \pm 1$ ml/mol for human $HbO_2$; and $\Delta H = 35.7 \pm 3.5$ kcal/mol, $\Delta V = 4.8 \pm 1$ ml/mol for bovine $HbO_2$, respectively. A possible explanation for the similar values between both human and bovine oxyhemoglobin is proposed. In addition, the PAC results for human $HbO_2$ and HbCO are compared and discussed.


*Keywords:* Hemoglobin; Photo-dissociation; Photoacoustic calorimetry; Enthalpy change; Volume change

Hemoglobin (Hb) is an instructive model for understanding the function of many regulatory proteins in the biological system. It is a tetramer (64,500) made up of two α-chains and two β-chains, and each of them carrying one hemo, respectively. It is well recognized that the physiological role of hemoglobin is to bind molecular oxygen in lungs and release it in tissues. The efficiency of this role stems from the cooperative nature of ligand binding in hemoglobin, where tetramer undergoes a transition between R (relaxed) and T (tensed) quaternary conformation of the protein, i.e., an allosteric process [1]. This suggests that the association and the dissociation processes for intermediates of hemoglobin are interdependent [2].

Transient kinetic technology is a useful approach to understand the association and dissociation reaction of ligand with hemoglobin. Although a great variety of modern experimental techniques, such as time-resolved UV–Vis absorption [3–9], IR [10–12], and Raman spectroscopies [13–17], have provided information about ligand recombination kinetics as well structures of intermediate species. How the protein structure controls the dynamics of energy for ligand binding and affects its catalytic function remain to be fully resolved.

Laser-induced time-resolved photoacoustic calorimetry (PAC) has received recognition as an effective technique for directly measuring both the thermodynamics and kinetic properties of photo-induced reactive intermediates. Based on the strong temperature dependence of the thermal expansion coefficient in aqueous solution, it is now feasible to measure the dynamics and magnitude of both enthalpy and molecular volume changes accompanied with the photo-induced ligand dissociation on the time scale of nanosecond to microsecond. It is applied to a variety of reactions encountered in organic, organo-metallic, and biological chemistry [18–22]. For the hemo-related biological systems, Peters et al. [23–25] successfully employed this method to investigate the association and dissociation reaction of CO ligand with myoglobin and hemoglobin, in which deoxymyoglobin and deoxyhemoglobin were transiently created through ligand photo-dissociation of MbCO and HbCO. Barker and Larsen [26] and Larsen [27] also examined volume and enthalpy profiles of CO

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ligand associated with a Fe(II) porphyrin hemo model system. In this paper, we first report the work of applying PAC method to study the photolysis of oxyhemoglobin ($HbO_2$) solutions for both human and bovine. The results of the enthalpy and volume changes for ligand dissociation of $HbO_2$ have been obtained and some influencing factors on them are also discussed.

## Experimental section

*Materials*. The solutions of Human hemoglobin (Sigma, USA) and bovine hemoglobin (Mingzhu Dongfeng Biological Technique, Shanghai, China) are prepared according to a previously published procedure [25]. They are first dialyzed against 50 mM phosphate buffer at pH 7.0 and then reduced with sodium dithionate ($Na_2S_2O_3$). Oxyhemoglobin was formed by bubbling $O_2$ through the solutions at 1 atm pressure. For PAC experiments, the initial and final concentration of oxyhemoglobin is about $3.5 \times 10^{-5}$ mol $L^{-1}$ by UV–Vis measurement, which indicates the content of $HbO_2$ in equilibrium component has no change almost. A solution of bromocresol purple (BCP) in 50 mM phosphate buffer, pH 7.0, is used as the calorimetric reference [25,28], which converts photon energy into heat entirely. Before the experiments are begun, the optical density of the sample and reference solutions at 532 nm are adjusted so as to be the same and measured by Shimadzu UV-3100 Spectrophotometer.

*PAC experiments*. The photoacoustic apparatus are same as that reported from our previous experiments and those of others [20–22,25]. In the present experiment, a Q-switched Nd:YAG laser (Continuum NP70) operating at 10 Hz, 532 nm, and pulsed width 8 ns, was used as the excitation source. The laser pulse energy was attenuated by means of a set of neutral density filters, which could change the pulse energy between 0 and 20 μJ. The laser beam diameter was fixed by a 0.9 mm pinhole, which could determine the time resolution to be equal to the travel time of the acoustic wave through the laser beam diameter. Temperature (7–22 °C) was kept constant within ±0.1 °C by using a thermostat and a thermoelement placed directly into the sample cell.

The acoustic wave induced in solution was detected by a 1.5 MHz PZT piezoelectric transducer. The signal was then amplified by a HP-8847F instrument and recorded by a digitizing oscilloscope (HP-54510B). The data were then transferred to a personal computer in which each acoustic wave was normalized to the laser pulse energy measured by a transient radiometer (Digi Rad R-752 and P-444).

*Data analysis*. According to the previous studies, the acoustic signal ($S$) results from the expansion or contraction, i.e., the volume changes of the irradiated sample, where the parameter $K$ is a function of the instrument response:

$$S = K\Delta V. \tag{1}$$

There are two contributions to the overall volume change. One is derived from the thermally induced volume change in the solution, $\Delta V_{th}$, which is related to the thermal expansion coefficient ($\beta$) for solvent and the heat capacity ($C_p$) of the solution. The other may arise from the volume change between products and reactants, $\Delta V_r$. Therefore, the acoustic wave amplitude $S$ is given by Eq. (2), where $\rho$ is the density of the solution, and $Q$ is the thermal energy released to the medium upon the decay process:

$$S = K(\Delta V_{th} + \Delta V_r) = K[(\beta/C_p\rho)Q + \Delta V_r]. \tag{2}$$

In this experiment bromocresol purple is used as the calorimetric reference. It converts the photon energy entirely into heat with no reaction volume change, i.e., $\Delta V_r = 0$. Therefore

$$S_{ref} = K(\beta/C_p\rho)E_{hv}. \tag{3}$$

The ratio of the acoustic wave amplitudes of the sample to the calorimetric reference is then defined as $\phi$, expressed as (4):

$$\phi = S/S_{ref} = (Q/E_{hv}) + \Delta V_r/[(\beta/C_p\rho)E_{hv}] \tag{4}$$

then (5) was given:

$$E_{hv}\phi = Q + \Delta V_r C_p\rho/\beta. \tag{5}$$

It is assumed that $Q$ and $\Delta V_r$ are independent of temperature. The intercept and slope of the linear plot of $E_{hv}\phi$ versus $C_p\rho/\beta$ at different temperatures yields $Q$ and $\Delta V_r$, respectively. However, the quantum yield $\Phi_d$ of the photo-induced chemical reaction must be taken into account for the evaluation of $\Delta H_R$ and $\Delta V_R$. Therefore, the overall enthalpy and volume change for reaction are determined according to Eqs. (6) and (7), respectively:

$$\Delta H_R = (E_{hv} - Q)/\Phi_d, \tag{6}$$

$$\Delta V_R = \Delta V_r/\Phi_d. \tag{7}$$

## Results

Photoacoustic signals of both $HbO_2$ and BCP over the energy range 0–20 μJ are measured. Taking signals at 18 °C for example, the linearity of the photoacoustic response with pulse energy (as shown in Fig. 1) reveals that there is no multi-photon effect in solutions under the experiment conditions. Photoacoustic signals of $HbO_2$ and BCP in the experimental window in 50 mM phosphate buffer, pH 7.0, are determined from 7 to 22 °C. As an example, Fig. 2 shows the signals at 13.3 and 20.2 °C, respectively. The similarity and no time shift of the waveshape for both sample and reference indicates that only one kinetic process exists in the experimental time window. Each photoacoustic waveform must be energy normalized, i.e., subtracted from the waveform baseline and divided the average laser pulse energy. After that, the ratio of signals of sample to reference, $\phi = S_{(HbO_2)}/S_{ref\ (BCP)}$ at the temperature ranging from 7 to 22 °C could be obtained.

Furthermore, the value of quantum yield for the photo-dissociation of human $HbO_2$ is 0.153, which could be deduced from the experiments of Duddell et al.

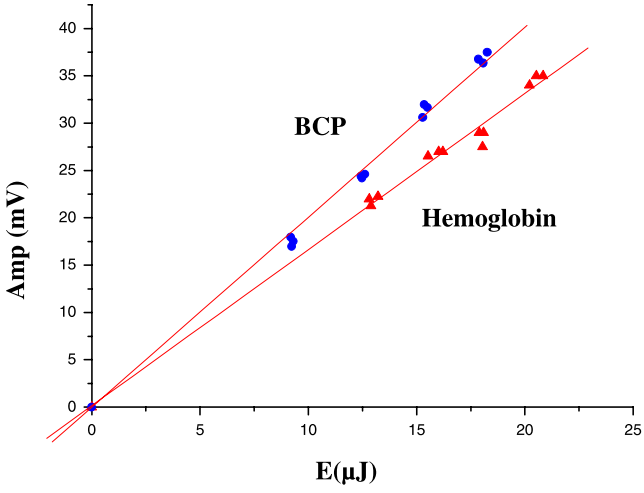


Fig. 1. Photoacoustic signals vs. pulse energy at 20 °C.

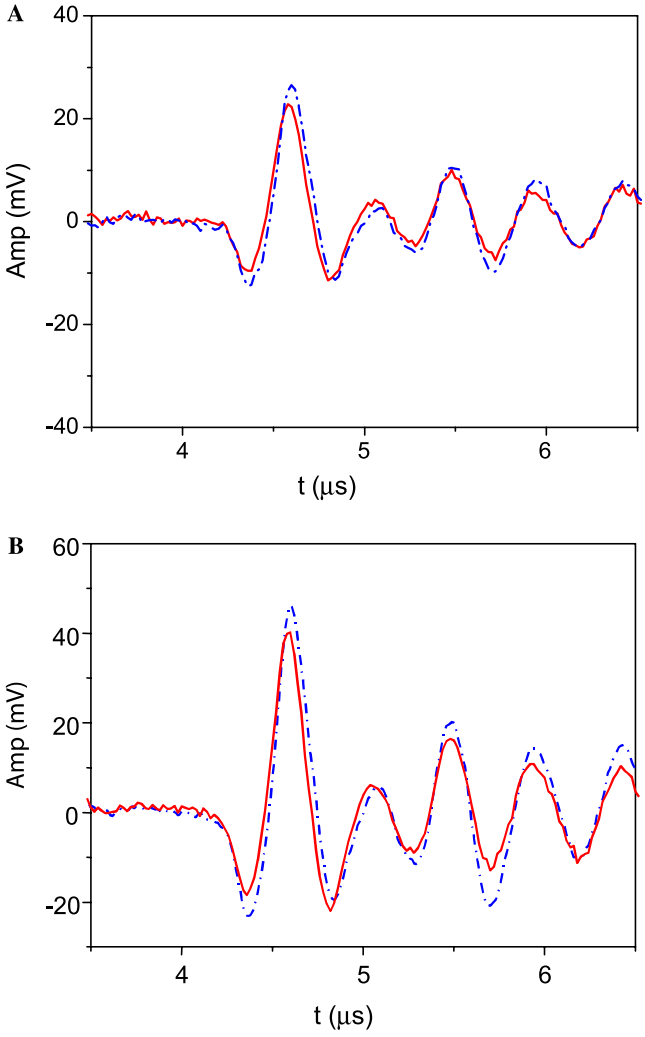


Fig. 2. (A) Photoacoustic signals vs. time of human $HbO_2$ (solid line) and BCP (dash dot line) at 13.3 °C. (B) Photoacoustic signals vs. time of bovine $HbO_2$ (solid line) and BCP (dash dot line) at 20.2 °C.

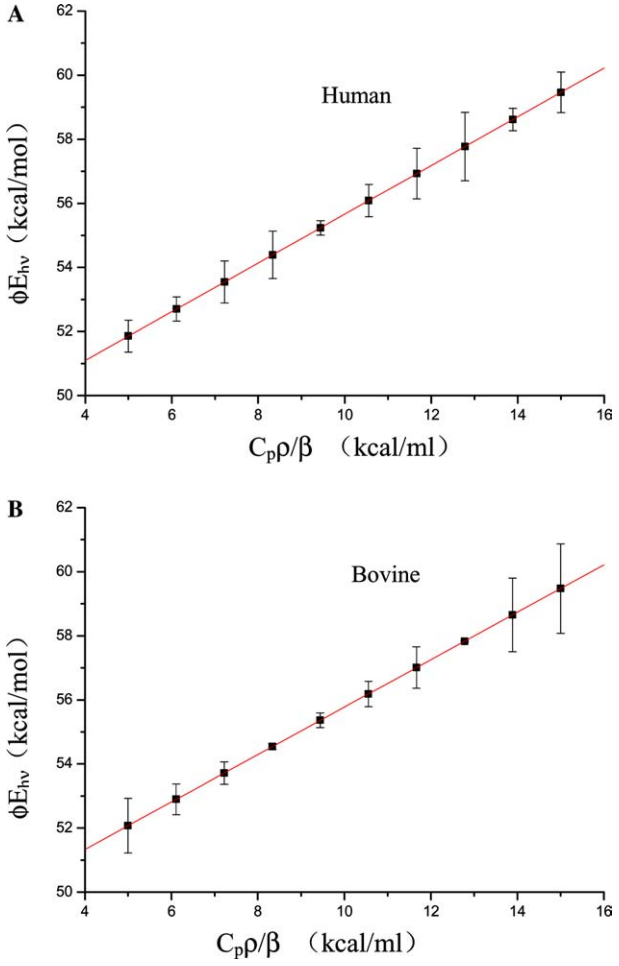


Fig. 3. Plot of $\phi E_{h\nu}$ vs. $C_p\rho/\beta$ for $HbO_2$ of (A) human and (B) bovine (in 50 mM phosphate buffer at pH 7.0).

[29], as well as Ghelichkhani et al. [30], in the nanosecond scale photolysis. While there is no quantum yield value for bovine to be referenced, we use the same value as for the human sample for the approximate calculation of bovine samples. This assumption can be rationalized by their high homologous structure characterizations (see the Discussion part below).

The plots by the PAC experiments for human $HbO_2$ and bovine are illustrated in Fig. 3. From the intercept and slope of $\phi E_{h\nu}$ versus $C_p\rho/\beta$, as well as $\Phi_d$, we obtained the values of $\Delta H = 37.8 \pm 3$ kcal/mol, $\Delta V = 5.0 \pm 1$ mL/mol for human $HbO_2$, and $\Delta H = 35.7 \pm 3$ kcal/mol, $\Delta V = 4.8 \pm 1$ mL/mol for bovine $HbO_2$, respectively.

Table 1
Comparing PAC results of $HbO_2$ (human and bovine) and HbCO (human)

| | $\Delta H_R$ (kcal/mol) | $\Delta V_R$ (mL/mol) | Reference |
|---|---|---|---|
| Human $HbO_2$ | $37.8 \pm 3$ | $5.0 \pm 1$ | This work |
| Bovine $HbO_2$ | $35.7 \pm 3$ | $4.8 \pm 1$ | This work |
| Human HbCO | $18.0 \pm 2.9$ | $23.4 \pm 0.5$ | [25] |

## Discussion

It can be seen from the data of Table 1 that the values of enthalpy and volume changes are very similar for photo-dissociation of human and bovine $HbO_2$. This seems reasonable since both of them are members of the same protein family. Comparing the sequence alignment of human and bovine hemoglobin (see Table 2) according to *Blast 2 Sequences* from the NCBI website (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html), we can find that both kinds of hemoglobin have high homology. So it is easy to explain the similar $\Delta H$ and $\Delta V$ values for them obtained in our PAC experiments.

The earlier interpretations of the events about dissociation of oxyhemoglobin are generally made using a hybrid of two models. One is the concerted model

Table 2
*Blast 2 Sequences* results of human and bovine hemoglobin

| | |
|---|---|
| Human | VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGK |
| Bovine | VLSAADKGNVKAAWGKVGGHAAEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGA |
| Human | KVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPA |
| Bovine | KVAAALTKAVEHLDDLPGALSELSDLHAHKLRVDPVNFKLLSHSLLVTLASHLPSDFTPA |
| Human | VHASLDKFLASVSTVLTSKYRVHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQ |
| Bovine | VHASLDKFLANVSTVLTSKY-RMLTAEEKAAVTAFWGKVKVDEVGGEALGRLLVVYPWTQ |
| Human | RFFESFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVD |
| Bovine | RFFESFGDLSTADAVMNNPKVKAHGKKVLDSFSNGMKHLDDLKGTFAALSELHCDKLHVD |
| Human | PENFRLLGNVLVCVLAHHFGKEFTPPVQAAYQKVVAGVANALAHKYHVLSPADKTNVKAA |
| Bovine | PENFKLLGNVLVVVLARNFGKEFTPVLQADFQKVVAGVANALAHRYHVLSAADKGNVKAA |
| Human | WGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGKKVADALTNAVAHV |
| Bovine | WGKVGGHAAEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGAKVAAALTKAVEHL |
| Human | DDMPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPAVHASLDKFLASVS |
| Bovine | DDLPGALSELSDLHAHKLRVDPVNFKLLSHSLLVTLASHLPSDFTPAVHASLDKFLANVS |
| Human | TVLTSKYRVHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPD |
| Bovine | TVLTSKY-RMLTAEEKAAVTAFWGKVKVDEVGGEALGRLLVVYPWTQRFFESFGDLSTAD |
| Human | AVMGNPKVKAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVC |
| Bovine | AVMNNPKVKAHGKKVLDSFSNGMKHLDDLKGTFAALSELHCDKLHVDPENFKLLGNVLVV |
| Human | VLAHHFGKEFTPPVQAAYQKVVAGVANALAHKYH |
| Bovine | VLARNFGKEFTPVLQADFQKVVAGVANALAHRYH |

Score = 983 bits (2542), expect = 0.0, identities = 492/574 (85%), positives = 526/574 (90%), gaps = 2/574 (0%).

(two-state), where the quaternary states of Hb comprise a high-affinity or relaxed state (*R*) and a low-affinity or tensed state (*T*) [31]. The other is the sequential model, that is, the association and disassociation of ligands are a sequence of four distinct equilibrium steps corresponding to the successive binding of ligands to deoxyhemoglobin and the three partially ligated Hb species [32]. According to a mount of X-ray data, Hoard–Perutz presented a "stereochemical trigger" hypothesis to understand the mechanism of cooperative oxygen binding to hemoglobin. There are three distinct changes involved in the process from the ligand-bound state to the ligand-free state of hemoglobin: (a) the movement of the iron atom relative to the heme plane, (b) the tertiary conformational change caused by this movement, and (c) the quaternary conformational change following the tertiary change [33–35]. Since 1980, a variety of laser photolysis techniques have been developed, so the dynamics of photo-dissociation for oxygen from $HbO_2$ have been clearly studied by the time-resolved transient spectra in femtosecond [36,37], picosecond [3–8], and nanosecond [30] time scales. It is recognized that after the rupture of the ligand–heme bond, the ligand can either rebind to hemo from within the protein (termed geminate recombination) or can escape into the surrounding solvent. In 1983, picosecond photolysis experiments for $HbO_2$ carried by Chernoff et al. [9] reveal that promptly following dissociation, the first geminate recombination process occurred with lifetimes of $200 \pm 70$ ps. In 1996, based on the nanosecond time-resolved absorption study of photolysis of $HbO_2$, Ghelichkhani et al. [30] demonstrated that the slow geminate recombination process was observed with a lifetime of 38 ns ± 10% and the tertiary relaxation occurs with a lifetime of 137 ns during the photolysis of $HbO_2$. Following those processes, there are two microsecond-scale ligand recombinations of $O_2$ to β and α chains of Hb with lifetimes of about 11 and 31 μs, respectively.

Considering the effective time scale of 100 ns–10 μs in our experiments, which were mainly determined by the pulse width of the exciting laser (8 ns) and the bandwidth of the transducer (1.5 MHz), the reported geminate recombination processes of 200 ps and 38 ns are both too fast to be resolved. Thus, the changes detected in the time window of this experiment may be caused by the tertiary relaxation of protein [30]. Additionally, the process of ligand recombinations in the microsecond could not be detected by our PAC instrument. Therefore, the enthalpy and volume changes associated with this formation correspond to the tertiary change in the R quaternary state without any quaternary structure changes, as described by Ghelichkhani et al. [30].

Ligand photo-dissociation of carboxyhemoglobin is another well-established model for understanding the association and dissociation reactions of ligands with hemoglobin. The dynamics of CO dissociation from R-state carboxyhemoglobin has been determined by transient flash photolysis [37–42]. This resulted in the conclusion that the first process for CO dissociation occurs within 300 fs [37]. The geminate pair lifetime in

R-state hemoglobin is of the order of about tens of nanoseconds at room temperature [38–40]. The second process involving a secondary geminate recombination and a tertiary relaxation at the hemo pocket occurs on the time scale of about 100 ns [3,16,40]. The third process occurring on the time scale of about 1 μs involved tertiary structure relaxations or a relaxation at the dimer–dimer interface, the first step in a $R \rightarrow T$ transition [13,15,38,41]. The fourth process that occurred with lifetime at 30–40 μs may be interpreted as the rate-limiting step of the quaternary structural changes [38,40,42]. The latter process, involving CO recombination to the R-state and T-state, occurred at 100 μs, and depends on the concentration of CO in solution [38,40]. Therefore, during the photo-dissociation process both oxyhemoglobin and carboxyhemoglobin experience analogous tertiary relaxation within the time scale windows of 100 ns–10 μs. This indicates that the values of enthalpy and volume changes for them would be similar.

When comparing the reported PAC experimental results of HbCO by Peters et al. [25] with those of our $HbO_2$, however, there are some differences between the two kinds of hemoglobin as shown in Table 1. The enthalpy change for the dissociation of oxyhemoglobin in the nanosecond time scale is about two times greater than that of carboxyhemoglobin. On the contrary, the volume change for the photo-dissociation of oxyhemoglobin is less than that of carboxyhemoglobin. We consider the main reason for this discrepancy is derived from the assumption about the quantum yield. Peters et al. assumed the quantum yield $Q = 1$ for photo-dissociation of carboxyhemoglobin, but a number of experiments reveal that as much as 50% of the photolyzed hemes may recombine geminately to ligand after a nanosecond laser pulse photo-dissociates the CO adduct [3,17,41]. In fact, most literatures accept the quantum yield of HbCO to be near 0.5 [3,17,41]. Thus, according to Eq. (6) the value of $\Delta H_{\mathrm{HbCO}}$ is about $36 \pm 3$ kcal/mol, while using $Q = 0.5$. Therefore, the enthalpy changes for photolysis of oxyhemoglobin and carboxyhemoglobin are comparable.

As for the $\Delta V$, it reflects both the solvation and the intrinsic molecular volume change and the latter is directly related to the structure change of reactive intermediate. The photo-dissociation of oxyhemoglobin with 100–500 ns lifetime was assigned to the process of a tertiary relaxation. Meanwhile, the same process for the carboxyhemoglobin was identified around 100 ns and extends into microsecond range. In the photolysis of ligand–hemoglobin, the driving force for tertiary relaxation derives from the available free energy, which initially induces a structure change at the heme and further effects the globin structure [33–35]. Although, there is no evidence to distinguish the degree of tertiary relaxation for both of oxyhemoglobin and carboxyhemoglobin in detail, we may infer that there is an analogous tertiary relaxation process during the photolysis which means that a similar protein structure change will contribute to the volume change observed by the PAC measurements.

It has been found that most oxygen rebinds to hemoglobin in the nanosecond to microsecond photolysis, while about half of carbon monoxide diffuses out of the protein matrix [8]. This result may throw a little light upon the differences in volume change values between HbCO in the literature and $HbO_2$ in our observation. In addition, a quantitative comparison of the volume changes observed between the oxyhemoglobin and carboxyhemoglobin is not significant, because this volume change represents only an increase of 0.02% of the total molecule volume of HbCO [25].

## Summary

In conclusion, we have successfully determined enthalpy and volume changes of oxyhemoglobin by time-resolved photoacoustic calorimetry. Their values are very similar for both human and bovine hemoglobin. Comparing the PAC data between ours and those reported for carboxyhemoglobin, we find that the enthalpy change of oxyhemoglobin is in concord with that for carboxyhemoglobin (when quantum yield is about 0.5). This is probably the reason that they undergo an analogous tertiary relaxation within the same time scale as in the PAC study.

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