VISIBLE FEL IRRADIATION EXPERIMENTS ON CARBONMONOXY HEMOGLOBIN*

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Abstract

Free electron laser (FEL) irradiation experiments with visible wavelengths were carried out: aliquots of carbonmonoxy hemoglobin A (HbACO) of *Podocnemis unifilis* (side-necked turtles, reptiles) were irradiated with 418 nm, 538 nm and 568 nm laser, respectively, and analyzed. These wavelengths were generated by the Laboratory for Electron Beam Research and Application (LEBRA), Nihon University, all of which were identical to maximum absorption spectra known as *Soret* band and *Q* bands (α and β) of vertebrate HbCO. After irradiation of up to approximately 50 J, the effect on the *Podocnemis* HbACO was evaluated by wavelength scan (350-700 nm regions) and Raman microscopy (532 nm excitation). We observed consistent shifts: from 418 nm to 410 nm for the *Soret* band, from 568 nm to 576 nm for α band, and from 538 nm to 541 nm for β band. These wavelength shifts might have been influenced by autoxidation of the *Podocnemis* HbACO during FEL irradiations. Raman analysis did not distinguish any structural differences in the target protein at any of the wavelengths.

INTRODUCTION

Living organisms have some colored macromolecules that, in general, play essential roles for their lives [1, 2]. As such a molecule, hemoglobin is designed to transport oxygen (O_2) to cells and tissue [3]. However, the binding and release of O_2 from hemoglobin is dependent on environmental conditions, such as the presence of carbonmonoxy molecules (CO). When CO molecules are present, O_2 binding to hemoglobin is compromised due to the stronger binding affinity of CO to the hemoglobin molecule [4]. The presence of carbonmnoxy hemoglobin can dramatically reduce the transport of oxygen to the tissues resulting in tissue hypoxia, therefore, the presence of carbonmonxy hemoglobin can be used as a diagnostic indicator in disease states characterized by tissue hypoxia [5]. Due to the devastating effects of HbCO in a living organism, it is essential to be able to detect changes in the hemoglobin molecule which reflect the binding of CO. FEL irradiation in one way in which these changes can be detected.

The LEBRA produces near infrared FELs including tunable wavelengths from 0.82 to 6.5 microns [6, 7]. The higher harmonics generated by means of the nonlinear optical crystals are also available with output energy of about 0.5 mJ/micro-pulse [8]. The LEBRA-FELs are of significant interest because we are able to adjust the wavelength exactly as needed. Also, The LEBRA-FELs cover wide ranges of wavelengths from visible and near infrared regions (350–6500 nm), expecting to unveil photochemical reactions of macromolecules, in particular colored molecules, even in living organisms.

During the course of biochemical and medical applications of the LEBRA-FELs, we initially used visible wavelengths of LEBRA-FELs to irradiate colored macromolecules such as carbonmonoxy hemoglobin (HbCO), whose maximum absorption spectra are known as *Soret* band (418 nm) and two weaker *Q* bands (α: 568 nm, β; 538 nm). After irradiation experiments, as a first step in interpreting the FEL irradiation effect, the target *Podocnemis* HbACO solution was investigated by means of visible scanning absorption spectroscopy and Raman microscopy. In this paper, we present the *Podocnemis* HbACO data obtained from the irradiation of visible FELs. We found differences in wavelength shifts and intensities of peaks in spectroscopic analyses. According to our best knowledge, irradiation experiments on HbCO with visible wavelengths have been reported [9, 10], however, there is no report that the HbCO has been irradiated by its maximum absorption wavelengths generated by exactly tuning the LEBRA-FELs as used in this study.

EXPERIMENTAL PROCEDURES

Preparation of Podocnemis HbACO

Blood of an adult side-necked turtle (*Podocnemis unifilis*) was drawn from the tail vein into a heparinized syringe using a 14 G needle. Red blood cells were obtained through centrifugation at 1000 rpm for 3 min, and the packed cells were subsequently rinsed several times with reptilian Ringer solution containing 50 mM EDTA. Finally, hemolysates were prepared in 1/15 M phosphate buffer, pH 7.0, and the membrane-free samples were obtained by centrifugation at 12,000 rpm for 30 min.

All samples were stored at 4 °C until ready for use. The samples were diluted with phosphate buffer at a concentration of approximately 11.5 mg/ml, which has an optical density of $A_{1cm,\ 538\ mn}$=5.0. For the preparation of the *Podocnemis* HbACO samples, CO gas (>99.95 %) was then passed for 2-3 hrs through a sealed 15 ml-tube containing the sample, which had been deoxygenated by gentle agitation in nitrogen atmosphere followed by the addition of solid sodium dithionite. To eliminate excess amount of reagents the *Podocnemis* HbACO was run a HiTrap™ desalting column (5 x 5 ml) on an AKTA purifier system (GE Healthcare Bio-Sciences AB,

*Work supported by Academic Frontier Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), Japan, 2005-2007
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Uppsala, Sweden). Finally, to confirm the presence of *Podocnemis* HbA not HbD, we examined the *Podocnemis* HbA fraction on Ether hydrophobic interaction column chromatography [11].

FEL Irradiation at 418 nm, 538 nm and 568 nm

Each of the FEL irradiation experiments on the *Podocnemis* HbACO were performed under the same conditions except for the wavelength which was used. The set up for LEBRA-FEL irradiation is shown in Fig. 1. Each of the three FEL beams was generated by means of forth higher harmonics [12]. The control experiments were done under the same conditions without FEL irradiation. Aliquots of 500 µl of Hb solution were placed in the wells of a 48-well plate (Sumitomo Co. Ltd., Japan) and while stirring, the samples were irradiated with each of the LEBRA-FELs laser beams.

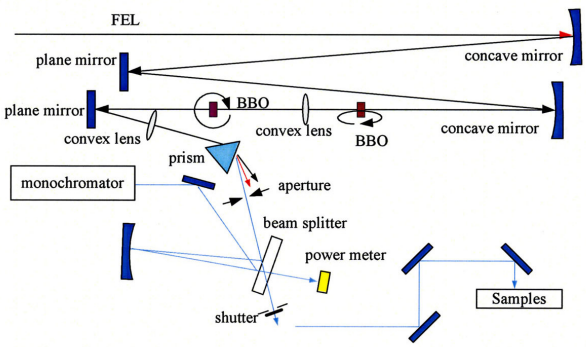

Figure 1: Experimental arrangement for the LEBRA-FELs irradiation.

Spectroscopic Measurements

In order to evaluate FEL irradiation experiments we conducted two spectroscopic analyses. The first spectroscopic analysis was scanning absorption spectroscopy (wavelength scan), which utilized an Ultraspec 2000 pro UV/Visible Spectrophotometer (Amersham Pharmacia Biotec, Sweden). The diagrams were printed out by a DPU-414 Thermal Printer (Seiko Instruments Inc., Chiba, Japan).

The second spectroscopic analysis was Raman microscopy. For this analysis, aliquots of 100 µl of *Podocnemis* HbACO samples with or without irradiation were placed in the wells of a U-plate 96-well (Greiner, Japan), and spectra were subsequently obtained on a Raman Microprobe (HoloLab^{TM} 5000R, Kaiser Optical Systems, Inc. USA) using 100 mW of 532 nm excitation. All the experiments were done at room temperature.

RESULTS AND DISCUSSION

The initially isolated *Podocnemis* HbA, designated as native HbA, shows three major absorption peaks in wavelength scan from 350 nm to 700 nm. These peaks are the Soret band (Fig. 2A) and two weaker bands (Fig. 2B), which are consistent to that which is reported in literature for animal hemoglobins [13]. When exposed to CO, the maximum absorption spectra of the *Podocnemis* HbACO shifted several nanometers from the original positions, verifying the presence of carbonmonoxy hemoglobin [14]. The *Podocnemis* HbACO has three maximum absorption spectra with 418 nm, 538 nm and 568 nm. Hence, in these FEL irradiation experiments, we investigated the wavelength shifts of these absorption spectra (418 nm, 538 nm, and 568 nm) as a function of irradiation effect of the visible FELs. In addition, these samples were analyzed by Raman microscopy.

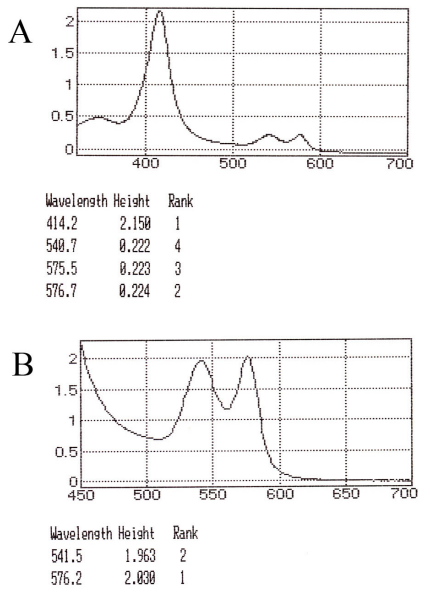

Figure 2: Typical absorption spectra of the *Podocnemis* HbA. A; The *Soret* band (414.2 nm). B; The *Q* bands (α band; 576.2 nm and β band; 541.5 nm).

Table 1A, 1B, and 1C summarize the wavelength shifts of maximum absorption spectra on FEL irradiation experiments with samples exposed to 418 nm, 538 nm, and 568 nm beams. As evident in the Table, the *Soret* band and *Q* bands of the *Podocnemis* HbACO changed. At a 568 nm beam with 3.4 Joule [J], the *Soret* band moved to 411 nm, α band of *Q* bands to 576 nm, and β band of Q bands to 541 nm. These values of wavelength shifts resemble those of the 414 nm irradiation as well as those of the 538 nm irradiation. Control experiments identified that the *Podocnemis* HbACO samples are influenced by the presence of oxygen which is supported by literature indicating that autoxidation of human HbCO occurs [14, 15].

Although the absorption spectra of the HbACO at the stated wavelengths showed blue-shifts and red-shifts (Table 1), the Raman spectra at the stated wavelengths were similar to each other. The Raman data obtained by the 538 nm FEL irradiation are shown in Fig. 3. Our data is remarkably similar to that of Strekas and Spiro [16] who have reported on Raman spectra of various derivatives of human Hbs, including HbCO. Of particular

interest are the bands observed for human HbCO at 1641 and 1589 cm^{-1} (these bands are missing for deoxyhemoglobin but present in oxyhemoglobin [16]). The two Raman bands were observed in all samples irradiated by different visible wavelengths of LEBRA-FELs (data not shown). We are currently engaged in examining differences in other Raman bands, but as yet, have failed to observe any Raman bands which characterize the structural difference of the *Podocnemis* HbAs when subjected to the FEL wavelengths.

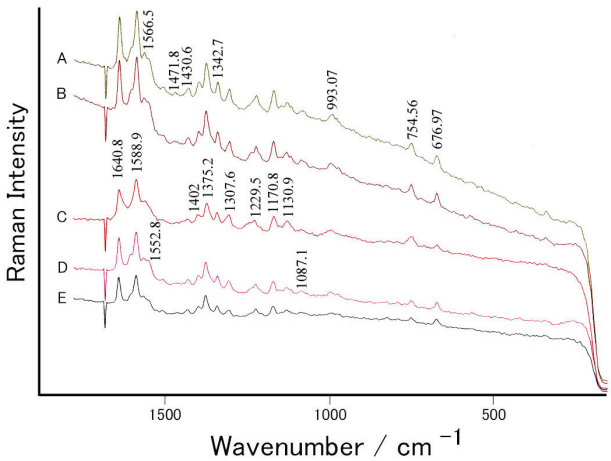

Figure 3: Raman spectra of the *Podocnemis* HbA and HbACO recorded with 532 nm excitation. All spectra of Hbs except for B and E were irradiated with the 538 nm laser. A; HbACO after 50-J irradiation. B; HbACO without irradiation. C; HbACO after 30-J irradiation. D; HbACO after 10-J irradiation. E; The native HbA without irradiation.

Table 1A: The Wavelength Shifts of the *Soret* and *Q* Bands (α and β) of the HbACO After 418 nm Irradiation

Original position of peak (nm)	Wavelength shift after FEL irradiation		
	1.6 [J]	5.0 [J]	8.0 [J]
418, *Soret*	418.3 (418.2)	415.9 (418.0)	410.4 (413.0)
539, β band	538.8 (538.8)	541.1 (539.4)	541.6 (541.3)
569, α band	569.5 (568.4)	575.5 (572.6)	576.7 (576.2)

Table 1B: The Wavelength Shifts of the *Soret* and *Q* Bands (α and β) of the HbACO After 538 nm Irradiation

Original position of peak (nm)	Wavelength shift after FEL irradiation		
	15 [J]	30 [J]	50 [J]
418, Soret	413.9 (416.8)	411.1 (412.0)	410.3 (412.3)
538, β band	540.4 (540.3)	540.9 (540.1)	540.7 (540.7)
568, α band	575.9 (575.2)	576.6 (576.1)	576.5 (576.2)

Table 1C: The Wavelength Shifts of the *Soret* and *Q* Bands (α and β) of the HbACO After 568 nm Irradiation

Original position of peak (nm)	Wavelength shift after FEL irradiation		
	0.36 [J]	3.0 [J]	3.4 [J]
418, Soret	417.4 (417.3)	414.0 (414.1)	410.6 (413.8)
538, β band	549.1 (539.2)	540.8 (540.4)	540.9 (541.8)
568, α band	570.1 (569.4)	575.9 (575.9)	575.7 (576.1)

The number in parentheses indicates the control without FEL irradiation.

CONCLUSIONS

The *Podocnemis* HbACO, as an example of colored bio-molecules, shows wavelength shifts on irradiation of visible LEBRA-FELs whose wavelengths are adjusting its maximum absorption spectra of the *Soret* band (418 nm) and two of the *Q* bands (538 and 568 nm). After irradiation, the structural changes of samples have been also examined by Raman microscopy but failed to show a difference due to similarity of Raman data from various derivatives of liganded hemoglobins, in particular HbCO and HbO_2. The LEBRA-FELs, however, are promising to provide a future tool probing photochemical reactions of living organisms.

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