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Original Article

Infrared Free Electron Laser or Polarized Ultraviolet Photolysis of Hierarchical and Chiral Components of Interleukin-6, Alanyl-Alanine and Alanine

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HIGHLIGHTS

- Interleukin 6 (IL-6) was decomposed by irradiation of IR-FEL (Infrared free electron laser).
- Parts of IL-6 (dipeptide and amino acid) was not decomposed by polarized IR-FEL nor UV (ultraviolet) light regardless of their chirality.
- Secondary structure of IL-6 was easier to be damaged by IR-FEL than covalent bonds.

ABSTRACT

Interleukin-6 (IL-6) could be decomposed by irradiation of IR-FEL (Infrared free electron laser). Using circularly polarized and other UV light and IR-FEL light, photolysis of hierarchical components of cast films of IL-6, namely deuterated aqueous solutions of enantiomers of dipeptide (*L*-alanyl-*L*-alanine (Ala-ala) or *D*-alanyl-*D*-alanine) and enantiomers of amino acid (*L*-alanine (Ala) or *D*-alanine) was investigated whether specific bonds can be broken by absorption of light (not due to heat). In addition, IR-FEL irradiation to powder as well as crystal structure determination for *L*-Ala and *D*-Ala at 173 and 293 K was also carried out to confirm reproducibility in the solid state about long-lasting controversy about Salam' hypothesis associated with chirality exhibiting structural phase transition at different temperature. Subunits of IL-6 (dipeptide and amino acid) could not be decomposed by polarized IR-FEL nor UV (ultraviolet) light regardless of their chirality. All experimental methods tested in this study failed to prove Salam's hypothesis, positively. Consequently, secondary structure of IL-6 was found to be easier to be damaged by IR-FEL than covalent bonds.

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Introduction

IL-6 was found as a cytokine to differentiate activated B cells into antibody-producing cells in 1986. IL-6 is

involved in a variety of biological events, including immune response, hematopoiesis, and acute phase response. IL-6 is known to be associated with various aspects, for example, as a prototypical cytokine featuring pleiotropic and redundant activity (Tanaka, 2015). Moreover, overproduction of IL-6 has been suggested to be involved in the development of some chronic inflammatory diseases and various diseases such as cancer (Hashizume et al., 2014) as well as the pathogenesis of rheumatoid arthritis (Nishimoto, 2008). In this context, other effective methods for decomposition of IL-6 may play an important role in medical application and must be developed even such a new strategy may be still hypothesis.

Recently, we have investigated the assistance effect of metal complexes adducts for protein molecules for damaging by IR-FEL (infrared free electron laser) (Onami et al., 2020). In this context, by using circularly polarized UV light and infrared free electron laser (IR-FEL) light, in this time, photolysis of hierarchical "chiral" components of cast films of a natural (*L*-) protein (*L*-6) will be attempted whether specific bonds can be broken by absorption of light in especially not due to heat (Fig. 1). Besides IL-6 case films, deuterated aqueous solutions of enantiomers of dipeptide (*L*-Ala-*L*-ala or *D*-Ala-*D*-ala) and enantiomers of amino acid (*L*-Ala or *D*-Ala) will be investigated as tested samples. In addition, IR-FEL irradiation to powder as well as crystal structure determination for *L*-Ala and *D*-Ala at 173 and 293 K was also carried out to confirm reproducibility in the solid state about long-lasting controversy about Salam' hypothesis associated with chirality exhibiting structural phase transition at different temperature.

By the way, so-called "Salam's hypothesis" (Salam, 1991) about chirality (Guijarro, 2009) has been in the limelight as an example of the ongoing controversy after the announcement of hypothesis verification and rebuttal in crystallographic studies again (Belo et al., 2018a; Belo et al., 2018b; Burgi et al., 2018). In recent years, especially, crystal structures of enantiomers of Ala (monomer of amino acid) newly by means of neutron crystallography and other methods were reported again (Wang et al., 2005) and a skeptical comment for it (Wang et al., 2000) and response as an objection (Sulivan et al., 2003) was repeatedly discussed in crystallographic journal. Originally, this hypothesis was proposed with the aid of superconductivity theory (Salam, 1991). In the crystal structure phase transition, the theory stated that enantiomers exhibit different transition behavior under various temperature due to an energy difference between them at sub-atomic level. There also are such controversy in spectroscopic studies (Gabuda et al., 2014; Kozlova et al., 2017).

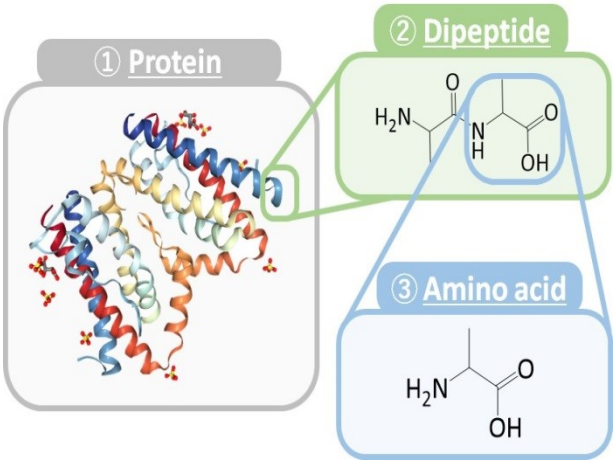

Figure 1. Outline of hierarchical components of (1) protein (IL-6), (2) dipeptide (Ala-ala), and (3) amino acid (Ala) for light irradiation.

Materials and Methods

Materials

The sources of reagents purchased are as follows. *L*-Ala (Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan), *D*-Ala (Tokyo Kasei Kogyo Co., Ltd.), *L*-Ala-*L*-ala (Sigma-Aldrich Japan, Tokyo, Japan), *D*-Ala-*D*-ala (Sigma-Aldrich Japan, Tokyo, Japan), Interleukin 6 (IL-6) (Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan), Deuterated water (D_2O) for NMR spectroscopy (MERCK (Cosmo Bio.)).

The sample for infrared free electron laser irradiation was prepared as follows. IL-6 is made by casting method. 0.0266 g of IL-6 was dissolved by adding 900μL of phosphate buffer, and 100 μL of methanol was added to the solution, followed by stirring until the protein was dissolved. This solution was taken with a 20μL pipette, dropped on a stainless plate, and left for about 1 hour to form a film.

The concentration of each heavy aqueous solution was prepared as follows. *L*-Ala in D_2O (5.0×10^{-3} M), *D*-Ala in D_2O (5.0×10^{-3} M), *L*-Ala-*L*-ala in D_2O (7.8×10^{-4} M), and *D*-Ala-*D*-ala in D_2O (7.8×10^{-4} M).

Light sources

The infrared free electron laser (IR-FEL) owned by Tokyo University of Science was used in this study; the oscillation wavelengths are ranged in the mid-infrared region (5.0-10.0 μm: 1,000-2,000 cm^{-1}). The time structure of the laser consists of macro and micro pulses: the macro pulse is composed of several hundreds of micro

pulses and has a duration of 2 μs and a repetition rate of 5 Hz. The half-width of the micro pulse is 1~2 ps and the interval of two consecutive pulses is 350 ps.

The UV lamp used for UV light irradiation was LA-410UV (HAYASHI-REPIC) and irradiated at 298K. The UV-visible absorption filter used was UTVAF-50S-34U (SHIGMAKOKI Co., Tokyo, Japan), and the λ / 4 wave plate was WPQ-3250-4M (SHIGMAKOKI Co., Tokyo, Japan).

Physical measurements

The instrument used for FT-IR measurement of alanine and alanyl-alanine in this study is FT-IR 4200 (Jasco Co., Tokyo, Japan). The analysis range was measured from 4000–400 cm^{-1} at 298 K. The infrared absorption spectra of protein samples were recorded by using an IRT-7000 (Jasco Co., Tokyo, Japan) and an FT/IR-6100 spectrometer (Jasco Co., Tokyo, Japan). IR-SSE analysis software (Jasco Co., Tokyo, Japan) was used for protein secondary structure analysis. In this program, a calibration curve was created based on the secondary structure data of 17 proteins before multicomponent analysis (partial least squares quantitative model) and saved as a standard data. The Amide I band was deconvoluted into four major bands: α-helix (1650-55 cm^{-1}), β-sheet (1625-40 cm^{-1}), and β-turn (1655-75 cm^{-1}), and other secondary structures (1645–50 cm^{-1}). The percentage of secondary structure was calculated using the peak intensities of these amide I bands to be averaged.

Electronic (UV-vis) spectra were obtained on a (Jasco Co., Tokyo, Japan) V-570UV-vis-NIR spectrophotometer in the range 400–200 nm at 298 K. ^{1}H-NMR spectra were recorded on a JEOL JMN-300 spectrometer (300 MHz) (JEOL, Tokyo, Japan).

X-ray crystallography

Colorless crystals of *L*-and *D*-ala were glued on top of a glass fiber rod. We coated with a thin layer of epoxy resin. Intensity data of diffraction were collected on a Bruker APEX2 CCD diffractometer (Bruker, Billerica, MA, USA) with graphite-monochromated Mo-Kα radiation (λ= 0.71073 Å) at 173 and 273 K. Data analysis was carried out with a SAINT program package (Bruker, Billerica, MA, USA). The structures were solved by direct methods with a SHELXS-97, expanded by Fourier techniques, and refined by full-matrix least-squares methods based on F^2 using a SHELXL-97 program (Sheldrick, 2008). An empirical absorption correction was applied by a program SADABS (Bruker, Billerica, MA, USA). All heavy atoms were refined using anisotropic thermal displacement parameters. All hydrogen atoms were located at geometrically calculated positions and they were refined using riding models. Crystallographic data are listed in Table 1. In this way, re-determination of crystal structures of *L*-and *D*-ala was carried out in order to confirm difference of phase transition according to Salam's hypothesis.

Table 1. Crystallographic data of *L*-and *D*-Ala at 173 and 273 K (re-determined in this work).

	***L*-Ala (173 K)**	***D*-Ala (173 K)**	***L*-Ala (273 K)**	***D*-Ala (273 K)**
Empirical formula	$C_3H_7NO_2$	$C_3H_7NO_2$	$C_3H_7NO_2$	$C_3H_7NO_2$
Crystal System	orthorhombic	orthorhombic	orthorhombic	orthorhombic
Space group	$P2_12_12_1$*(#14)*	$P2_12_12_1$*(#14)*	$P2_12_12_1$*(#14)*	$P2_12_12_1$*(#14)*
Z	4	4	4	4
a/Å	5.785(4)	5.7957(11)	5.7851(3)	5.7889(5)
b/Å	5.980(4)	5.9766(11)	6.0330(3)	6.0399(7)
c/Å	12.311(10)	12.297(2)	12.3550(6)	12.3639(14)
V/$Å^3$	425.9(5)	425.22(14)	431.21(4)	432.30(8)
ρ(calc)/gcm^{-3}	1.390	1.389	1.372	2.738
μ /mm^{-1}	0.116	0.116	0.115	0.228
F(000)	192	192.0	192.0	384
Goodness of fit	1.091	1.126	1.182	1.065
R1 [$I>2\sigma(I)$]	0.0381	0.0342	0.0296	0.0584
wR2	0.0944	0.0886	0.0803	0.1566
Flack parameter	-0.3(7)	-0.1(6)	-0.3(3)	-0.6(4)

Results and Discussion

IR-FEL irradiation of IL-6

The irradiation wavelength of IR-FEL for IL-6 was determined to be 6.05 μm (Amide I) by FT-IR measurement. The sample of IL-6 as cast film was prepared as described above and was irradiated by IR-FEL light of 6.05 μm for 5, 10, 20, and 30 min, respectively, and the results of FT-IR infrared microscopy (IRM) analyzed by protein secondary structure analysis (Table 2 and Fig. 2). However, we have previously revealed that human serum albumin (HSA) protein including metal complexes could not be decomposed even by irradiation of synchrotron UV light (Tsuda et al., 2016). In contrast to the previous study on HAS exhibiting changes in secondary structures, considerable degradation of the protein molecule could be obtained except for slight changes of α-helix moiety, as a result of the IR-FEL irradiation under this condition. The structure of IL-6, which is a pleiotropic cytokine with a variety of stimulatory effects on hematopoietic cells, is known to be composed of a four helix bundle linked by loops and an additional mini-helix.

According to 1ALU of PDB (Somers et al., 1997) sequence of IL-6 was as follows in which several A (ala) and one AA (ala-ala) residues were contained.

MAPVPPGEDSKDVAAPHRQPLTSSERIDKQIRYIL
DGISALRKETCNKSNMCESSKEALAENNLNLPKM
AEKDGCFQSGFNEETCLVKIITGLLEFEVYLEYLQ
NRFESSEEQARAVQMSTKVLIQFLQKKAKNLDAI
TTPDPTTNASLLTKLQAQNQWLQDMTTHLILRSF
KEFLQSSLRALRQM

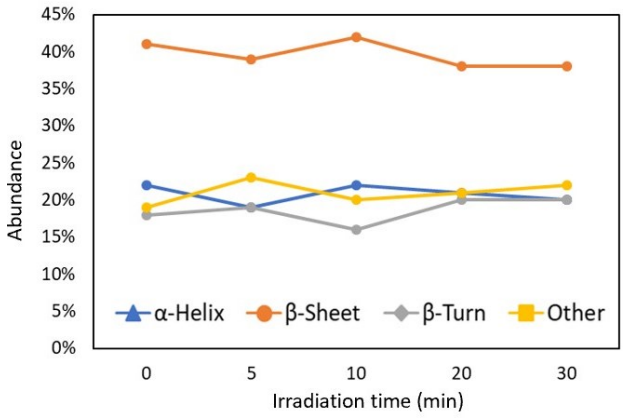

Figure 2. The results of protein secondary structure analysis of a cast film of IL-6 after IR-FEL irradiation at 6.05 μm.

Table 2. The results of protein secondary structure analysis of a cast film of IL-6 after IR-FEL irradiation at 6.05 μm.

time (min)	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Other (%)
0	22	41	18	19
5	19	39	19	23
10	22	42	16	20
20	21	38	20	21
30	20	38	20	22

Thermal decomposition of IL-6

In the thermal decomposition of IL-6, it was left at room temperature for 60 min. Generally, IL-6 was handled or carried at low temperature except for this operation during this study. From the results of the protein secondary structure analysis, the degradation of IL-6 was confirmed even after 20 min. Because molecular structure of IL-6 is composed of four α-helix moieties mainly, steric structure associated with α-helix moieties was damaged seriously. Therefore, it was experimentally proved that the mechanism of degradation of proteins in the case of IR-FEL (vibrational excitation) was different from thermal effects (in other words, high temperature even by IR light potentially) (Table 3 and Fig. 3).

Table 3. The results of protein secondary structure analysis of a cast film of IL-6 after thermal decomposition at room temperature (273 K).

time (min)	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Other (%)
0	3	35	30	32
5	4	36	29	31
10	3	35	30	32
15	3	38	28	31
20	0	33	33	34
25	3	35	31	31
30	9	29	30	32
35	14	28	28	30
40	9	26	32	33
45	9	31	30	30
50	10	33	28	29
55	12	31	27	30
60	8	34	28	30

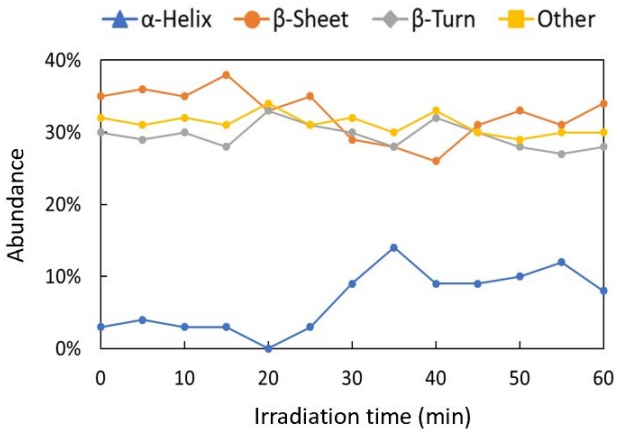

Figure 3. The results of protein secondary structure analysis of a cast film of IL-6 after thermal decomposition at room temperature (273 K).

IR-FEL irradiation of Ala-ala

Secondly, dipeptide moiety (Ala-ala) was investigated to examine possibility of photolysis at peptide H-N-C=O bonds by not only IR-FEL irradiation but also UV light irradiation. According to IR spectra of (Fig. 4), the wavelength corresponding to 6.49 μm C-N single bond in peptide bonds was selected as wavelength of IR-FEL irradiation for Ala-ala solutions. Indeed, there have been reposted several examples of photochemical reactions induced by circularly polarized UV light such as enantioselective excitation (Tang et al., 2011), enantioselective photoisomerization of N=N bonds (Hashim et al., 2019), dynamic control and amplification around C=C bonds (Huck et al., 1996), chiral C-C polymerization of achiral monomers (Manaka et al., 2006). However, only a few studies of chiral metamaterials using far-infrared teraheltz light have been reported (Yogesh et al., 2015).

^{1}H-NMR measurement as D_2O solutions was performed to confirm the structural change of the molecule before and after irradiation. ^{1}H-NMR of Alanyl-alanine before IR-FEL irradiation is as follows. In the IR-FEL irradiation, the heavy aqueous solution was irradiated with linearly polarized light and right and left circularly polarized light in 5, 10, 20, and 30 min. In the case of linearly polarized light irradiation, irradiation was performed at 3.0 Hz, 3.3 mJ / pulse at a wavelength of 6.49 μm. For left and right circularly polarized light irradiation, irradiation was performed at a wavelength of 6.49 μm at 3.0 Hz, 0.5 mJ / pulse. The following shows the results of ^{1}H-NMR after irradiation of linearly polarized light (Table S1), right circularly polarized light (Table 4), and left circularly polarized light (Table S2). As a result of the irradiation, no structural change could be confirmed by ^{1}H-NMR.

Table 4. Summary of ^{1}H-NMR data for Ala-ala after RCP IR-FEL irradiation (6.49 μm, 3.0 Hz, 3.3 mJ/pulse). In tables of NMR results, dq, d, and m denote double-doublet, doublet, and multiplet splitting, respectively.

Time (min)	Chemical shift	J-coupling	J (Hz)	proton	integrated value	Chemical shift	J-coupling	J (Hz)	proton	integrated value
	L-ala-*L*-ala/D_2O (7.8×10^{-3} M)					*D*-ala-*D*-ala/D_2O (7.8×10^{-3} M)				
0	3.86-4.00	m	-	2H	2.00	3.85-4.00	m	-	2H	2.00
	1.38	d	7.2	3H	3.02	1.38	d	6.9	3H	3.01
	1.18	d	7.6	3H	3.03	1.18	d	7.2	3H	3.00
5	3.91	dq	24.8, 7.2	2H	2.00	3.91	dq	24.8, 7.2	2H	2.00
	1.36	d	7.2	3H	3.01	1.36	d	7.2	3H	3.03
	1.17	d	7.2	3H	3.08	1.17	d	7.2	3H	3.05
10	3.91	dq	24.3, 7.2	2H	2.00	3.91	dq	24.6, 7.2	2H	2.00
	1.36	d	7.2	3H	2.97	1.36	d	7.2	3H	3.00
	1.17	d	7.6	3H	3.04	1.17	d	7.2	3H	3.01
20	3.91	dq	24.6, 7.2	2H	2.00	3.91	dq	25.0, 7.2	2H	2.00
	1.36	d	7.2	3H	3.03	1.36	d	7.2	3H	3.01
	1.17	d	7.2	3H	3.00	1.17	d	7.6	3H	3.00
30	3.91	dq	24.1, 7.2	2H	2.00	3.91	dq	24.8, 7.2	2H	2.00
	1.36	d	7.2	3H	3.06	1.36	d	6.9	3H	3.01
	1.17	d	7.2	3H	3.06	1.17	d	7.2	3H	3.00

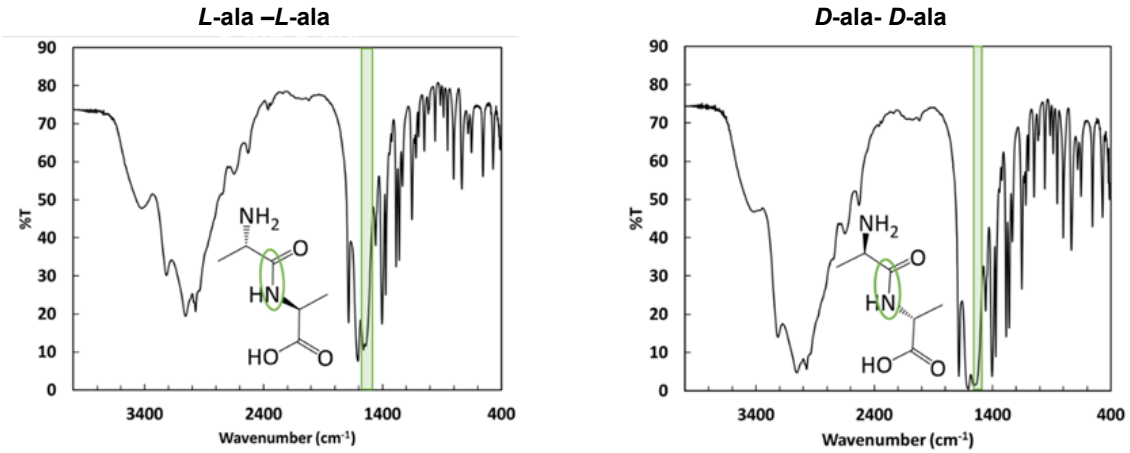

Figure 4. FT-IR of spectra of Ala-ala and selected irradiation wavelengths (green marked).

UV irradiation of Ala-ala

Next, dipeptide molecule is examined. Based on the results of UV-vis measurement, the irradiation range of UV light was determined to be around 220 nm according to absorption maximum peaks (Fig. 5). Similar ^{1}H-NMR results after UV light irradiation are shown (Tables 5, S3 and S4). Non-polarized light was irradiated for 15, 30 and 60 min, and left and right circularly polarized light was irradiated for 30 min. No structural change due to irradiation was observed here. According to Salam's hypothesis, parity-violating energy difference of transition energy between enantiomers should emerge (Letkhov 1975; Quack 2002), though it could not be detected in both cases of IR and UV light. It should also be noted that covalent peptide bonds could not be broken by irradiation of not only UV light but also IR light even as solutions, in contrast to the secondary structure of protein as cast films.

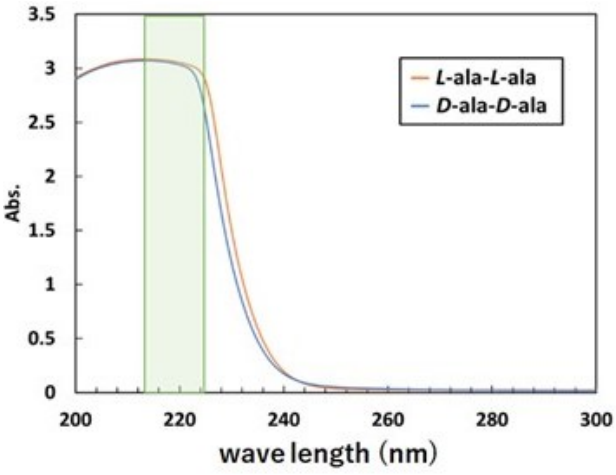

Figure 5. UV-vis spectra of Ala-ala and selected irradiation wavelengths (in nm, green marked).

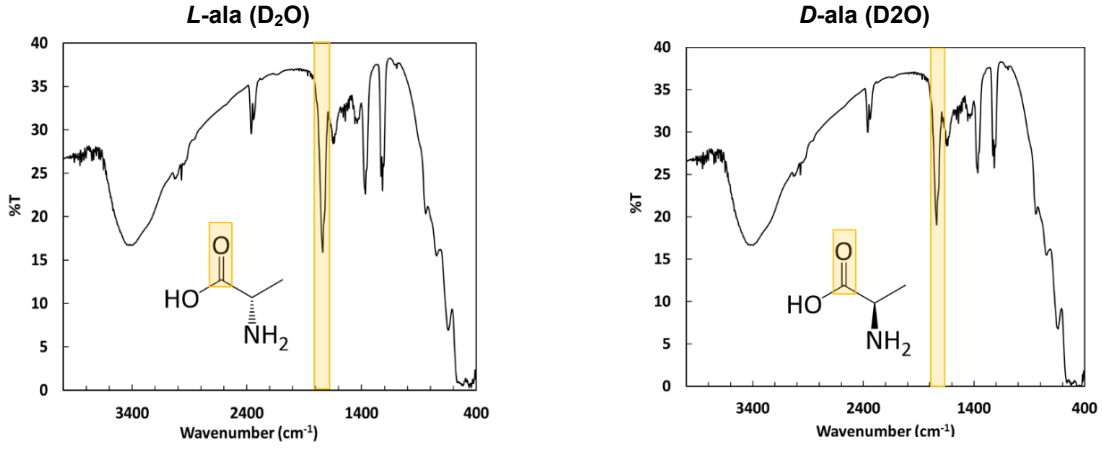

Figure 6. FT-IR of spectra of Ala and selected irradiation wavelengths (yellow marked).

Table 5. Summary of ^{1}H-NMR data for Ala-ala after RCP UV irradiation. In tables of NMR results, dq, d, and m denote double-doublet, doublet, and multiplet splitting, respectively.

time (min)	Chemical shift	J-coupling	J (Hz)	proton	integrated value	Chemical shift	J-coupling	J (Hz)	proton	integrated value
	L-ala-*L*-ala/D_2O (7.8×10^{-3} M), UV NL					*D*-ala-*D*-ala/D_2O (7.8×10^{-3} M), UV NL				
15	3.86-4.00	M	-	2H	2.00	3.85-4.00	m	-	2H	2.00
	1.38	D	7.2	3H	3.04	1.37	d	7.2	3H	3.02
	1.18	D	7.2	3H	3.03	1.18	d	7.2	3H	3.00
30	3.86-4.00	m	-	2H	2.00	3.85-4.00	m	-	2H	2.00
	1.38	d	7.2	3H	3.00	1.38	d	7.2	3H	3.04
	1.18	d	7.2	3H	3.09	1.18	d	7.6	3H	3.06
60	3.86-4.00	m	-	2H	2.00	3.85-4.00	m	-	2H	2.00
	1.37	d	6.9	3H	3.00	1.37	d	7.2	3H	1.37
	1.18	d	7.2	3H	3.01	1.18	d	7.2	3H	1.18
	L-ala-*L*-ala/D_2O (7.8×10^{-3} M), UV LCP					*D*-ala-*D*-ala/D_2O (7.8×10^{-3} M), UV LCP				
30	3.86-4.00	m	-	2H	2.00	3.85-4.00	m	-	2H	2.00
	1.38	d	6.9	3H	3.05	1.38	d	6.9	3H	3.05
	1.18	d	7.2	3H	3.00	1.18	d	7.2	3H	3.06
	L-ala-*L*-ala/D_2O (7.8×10^{-3} M), UV RCP					*D*-ala-*D*-ala/D_2O (7.8×10^{-3} M), UV RCP				
30	3.86-4.00	m	-	2H	2.00	3.85-4.00	m	-	2H	2.00
	1.38	d	6.9	3H	3.00	1.37	d	7.2	3H	3.02
	1.18	d	7.2	3H	3.03	1.18	d	7.2	3H	3.00

IR-FEL and irradiation of Ala

Thirdly, the wavelength corresponding to 6.49 μm covalent C = O double bond was selected from the results of the FT-IR measurement (Fig. 6) and around 220 nm from the UV-vis spectrum (Fig. 7) for the D_2O solution of Ala prepared similar to Ala-ala above.

In the IR-FEL irradiation experiments, Ala in D_2O was irradiated with linearly polarized light and right and left circularly polarized light in 5, 10, 20, and 30 min. For linearly polarized light irradiation, irradiation was performed at a wavelength of 5.75 μm at 5.0 Hz, 5.4 mJ/pulse (but in vain). For left (not shown) and right circularly polarized light irradiation, irradiation was performed at 5.0 Hz, 1.0 mJ / pulse at a wavelength of 5.75 μm. The results of ^{1}H-NMR after irradiation of right circularly polarized IR-FEL and UV light (Tables 6 and 7) are summarized. Besides D_2O solutions, similar experiments were also carried out for Ala in the solid states (Fig. S1, Tables S5 and 6). As a result of the irradiation, for all cases, no structural changes could be confirmed by ^{1}H-NMR similar to Ala-ala.

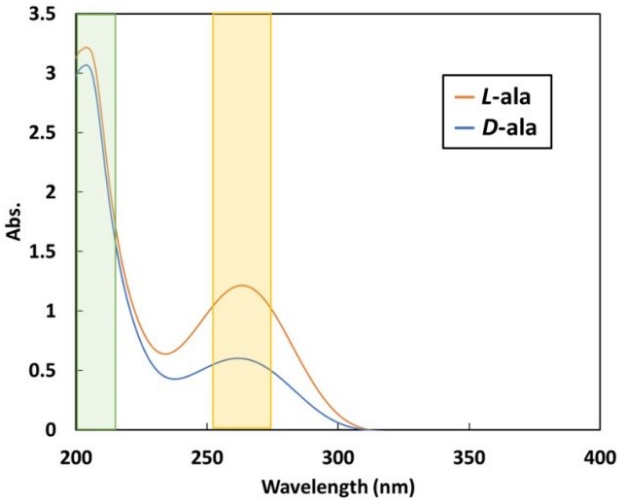

Figure 7. UV-vis spectra of Ala-ala and selected irradiation wavelengths (in nm, green marked).

Table 6. Summary of ^{1}H-NMR data for Ala after RCP (right circular polarized) IR-FEL irradiation (6.49 μm, 3.0 Hz, 3.3 mJ/pulse). In tables of NMR results, dq, d, and m denote double-doublet, doublet, and multiplet splitting, respectively.

Time (min)	Chemical shift	J-coupling	J (Hz)	proton	integrated value	Chemical shift	J-coupling	J (Hz)	proton	integrated value
	L-Ala/D_2O (7.8×10^{-3} M)					*D*-Ala /D_2O (7.8×10^{-3} M)				
0	3.86-4.00	m	-	2H	2.00	3.85-4.00	m	-	2H	2.00
	1.38	d	7.2	3H	3.02	1.38	d	6.9	3H	3.01
	1.18	d	7.6	3H	3.03	1.18	d	7.2	3H	3.00
5	3.91	dq	24.8, 7.2	2H	2.00	3.91	dq	24.8, 7.2	2H	2.00
	1.36	d	7.2	3H	3.01	1.36	d	7.2	3H	3.03
	1.17	d	7.2	3H	3.08	1.17	d	7.2	3H	3.05
10	3.91	dq	24.3, 7.2	2H	2.00	3.91	dq	24.6, 7.2	2H	2.00
	1.36	d	7.2	3H	2.97	1.36	d	7.2	3H	3.00
	1.17	d	7.6	3H	3.04	1.17	d	7.2	3H	3.01
20	3.91	dq	24.6, 7.2	2H	2.00	3.91	dq	25.0, 7.2	2H	2.00
	1.36	d	7.2	3H	3.03	1.36	d	7.2	3H	3.01
	1.17	d	7.2	3H	3.00	1.17	d	7.6	3H	3.00
30	3.91	dq	24.1, 7.2	2H	2.00	3.91	dq	24.8, 7.2	2H	2.00
	1.36	d	7.2	3H	3.06	1.36	d	6.9	3H	3.01
	1.17	d	7.2	3H	3.06	1.17	d	7.2	3H	3.00

Table 7. Summary of ^{1}H-NMR data for Ala-ala after RCP (right circular polarized), LCP (left circular polarized), and LP (linear polarized) UV irradiation. In tables of NMR results, dq, d, and m denote double-doublet, doublet, and multiplet splitting, respectively.

time (min)	Chemical shift	J-coupling	J (Hz)	proton	integrated value	Chemical shift	J-coupling	J (Hz)	proton	integrated value
	L-ala/D_2O (5.0×10^{-4} M), UV LP					*D*-ala/D_2O (5.0×10^{-4} M), UV LP				
15	3.63	q	7.2	1H	1.00	3.62	q	7.2	1H	1.00
	1.32	d	7.2	3H	3.01	1.32	d	7.2	3H	3.00
30	3.62	q	7.3	1H	1.00	3.62	q	7.2	3H	1.00
	1.32	d	7.2	3H	3.05	1.32	d	7.2	3H	3.09
60	3.61	q	7.2	1H	1.00	3.61	q	7.2	1H	1.00
	1.31	d	7.2	3H	3.04	1.31	d	7.2	3H	3.00
	L-ala/D_2O (5.0×10^{-4} M), UV LCP					*D*-ala/D_2O (5.0×10^{-4} M), UV LCP				
30	3.62	q	7.2	1H	1.00	3.62	q	7.2	1H	1.00
	1.32	d	7.2	3H	3.02	1.32	d	7.2	3H	3.08
	L-ala/D_2O (5.0×10^{-4} M), UV RCP					D-ala/D_2O (5.0×10^{-4} M), UV RCP				
30	3.62	q	7.2	1H	1.00	3.62	q	7.2	1H	1.00
	1.32	d	7.2	3H	3.02	1.32	d	7.2	3H	3.01

Redetermination of crystal structures of Ala

According to Salam's hypothesis, the crystal structure was changed around 250K, and the crystal structure of *L*-Ala and *D*-Ala was analyzed again at 173 K and 293 K (Fig. 8), namely temperature of low and high temperature phases. Indeed, there are many reports on crystal structures of various types of Ala by neutron diffraction (Wilson et al., 2005) from the first X-ray report (Simpson et al., 1966) and also for Ala-ala (Fletterick et al., 1971). However, no detectable differences due to structural changes was found in our results (Table 8).

Table 8. Selected bond lengths (Å) and angles (°) for Ala.

Geometries	Conditions			
	L-ala (173 K)	*D*-ala (173 K)	*L*-ala (273 K)	*D*-ala (293 K)
O1-C1	1.250(2)	1.2489(16)	1.2448(16)	1.26(6)
N1-C2	1.492(2)	1.4907(18)	1.4890(17)	1.50(6)
C1-O2	1.262(2)	1.2603(17)	1.2573(16)	1.24(6)
C1-C2	1.532(2)	1.5328(19)	1.5331(17)	1.53(6)
C2-C3	1.525(2)	1.5255(19)	1.5225(19)	1.53(7)
O1-C1-O2	125.87(16)	125.85(13)	125.78(12)	125.50(4)
O1-C1-C2	118.32(14)	118.16(12)	118.30(11)	118.70(4)
O2-C1-C2	115.80(15)	115.99(11)	115.92(11)	115.80(4)
N1-C1-C2	109.92(15)	110.05(11)	110.01(10)	109.90(4)
N1-C2-C3	109.77(16)	109.68(12)	109.80(11)	109.9(4)
C1-C2-C3	111.27(15)	111.30(11)	111.10(14)	111.30(4)

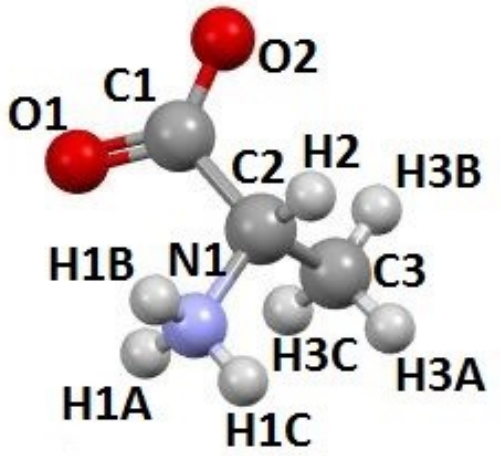

Figure 8. Crystal structures of *L*-Ala with atomic labels.

During summarizing some review articles about crystallographic studies, the authors have sometimes encountered "problematic crystal structures" ascribing to referring an original report containing wrong information or historical development of experimental techniques. The former is a simple cause, but it may have a significant impact on subsequent discussions. On the other hand, the latter is not for this case using conventional instruments (without progress of research tools).

According to our interest in chemical crystallography about chirality such as chiral crystallization, spontaneous resolution, and chiral molecular recognition, herein, we have carried out re-determination of crystal structures of alanine enantiomers (space group $P2_12_12_1$ at 173 and 293 K, respectively. Thus, we have confirmed that there are little differences among them. Additionally, light irradiation to break specific chemical bonds also exhibited similar results for alanine enantiomers.

Conclusion

For the purpose of future medical application, as a method of selectively destroying proteins that cause diseases, IR-FEL irradiation was used to study IL-6. We succeeded in damaging the secondary structure of IL-6 by IR-FEL irradiation under this condition for samples (concentration of solutions, films, or crystals) and light (wavelength or intensity). This was also found to behave differently than in the case of heat denaturation for the first time. In addition, hypotheses related to the origin of chirality, in comparison with UV irradiation to elucidate the mechanism of destruction, other attempts were made to break peptide bonds in dipeptide and cleave the carbonyl group of the amino acid, but no change was observed for the cases. Therefore, IR-FEL irradiation revealed that the secondary structure of proteins is more susceptible than the covalent bonds of their components clearly.

Ethical Statement

This article does not contain any studies involving animals or human participants performed by any of the authors.

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Competing Interests

The authors declare that they have no conflict of interest.

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Authors' Contribution

This work was carried out in collaboration with all the authors. T. Akitsu designed the study, N. Fujisawa, Y. Onami, and T. Kawasaki performed the experiments, N. Fujisawa and T. Akitsu wrote the draft of the manuscript, and T. Haraguchi revised the manuscript. K. Tsukiyama supervised the FEL-TUS, IR-FEL facility.

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