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Iron release analyses from ferritin by visible light irradiation

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Abstract

We investigated the iron release from ferritin by irradiation from a white fluorescent light in the absence or presence of ADP. Irradiation of a ferritin solution at 17,000 *lx* in the absence of ADP slightly induces iron release from ferritin but only at acidic pH conditions (pH 5.0 or pH 6.0). Irradiation in the presence of ADP markedly enhances iron release from ferritin under the same conditions. In the absence of irradiation, the iron release from ferritin was low even in the presence of ADP. The induction of the iron release by irradiation in the presence of ADP was also affected by various factors such as irradiation dose and acidity, but not temperature (4–47°C), oxygen concentration, or free radical generations during the irradiation. The iron release during the irradiation ceased to increase by turning off the light and was found to increase again after additional irradiation. These results suggest that visible light directly induces iron release from ferritin via the photoreduction of iron stored inside ferritin.

Keywords: *ADP, ferritin, iron release, visible light*

Introduction

Free radical mechanisms have been implicated in many diseases. Since irradiation generates a variety of oxygen species and free radicals, there have been many studies to demonstrate that free radical mechanisms are involved in photic injuries to the ocular and cutaneous tissues such as the retina [1–3], lens [4], and skin [5]. The elucidation of the mechanism has attracted much attention, as the ocular tissues such as the retina contain unsaturated fatty acids that are exposed to light for a lifetime. It is well known that these unsaturated fatty acids are susceptible to lipid peroxidation [6]. Therefore, many investigations have been carried out to research the relationship between free radical mechanisms and retinal photic disorders, focusing on the age-related macular degeneration [2]. On the other hand, when rats are exposed to intense light for a long period of time, the photic changes in the retina are inhibited by the administration of an iron chelator, desferrioxamine [1], or hydroxyl radical scavenger, dimethylthiourea [3], suggesting the implication of both iron and hydroxyl radical (·OH) in the development of some retinal photic disorders. In human skin, ferritin is regularly present in the basal layer of unexposed epidermis *in vivo*. Following ultraviolet-A (UV-A) exposure, ferritin increased both in epidermal and in dermal tissue [7]. Thus, it is considered that iron plays an important role in the mechanism of photic injury of

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superficial tissues like eyes and skin by visible light and ultraviolet ray.

While iron is present as one of the essential vital element in tissues and cells in the entire body, iron is known to be a crucial factor for the generation of reactive oxygen radicals, such as $^{\cdot}OH$ by way of the Fenton reaction. The hydroxyl radical is strongly connected with the lipid peroxidation in membranes which causes damage to tissues and cells. However, a large proportion of tissue non-haem iron is also stored in the stable form of ferritin and haemosiderin, which are not connected in most cases with $^{\cdot}OH$ formation [8].

Ferritin is a very important protein for iron storage, existing as ferrihydrite [9]. Unfortunately, the exact biochemical mechanism of the iron release from ferritin is not fully understood. Several studies indicate that the iron release from ferritin is induced by biological molecules such as cysteine, glutathione [10], and ascorbic acid [10–12]. Since superoxide anion radical ($O_2^{\cdot-}$) is also known to induce the iron release from ferritin, the harmful effects of the inflammation on tissues are not only due to the toxicity of $O_2^{\cdot-}$ but also $^{\cdot}OH$ generated via the Fenton reaction due to the released iron from ferritin by $O_2^{\cdot-}$ [13].

Recent studies have shown that UV-A irradiation of the ferritin solution induces the iron release depending on the wavelength and pH values without any change in the amino acid component of the apo-protein [14,15]. The iron release from ferritin has also been reported by gamma-ray irradiation [16]. However, no report has been published to investigate whether visible light can induce the iron release from ferritin or not. On the other hand, efficient iron release from ferritin requires the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) [17]. We used adenosine-5′-diphosphate (ADP) as the chelator of ferrous iron. ADP exists at a high level in all types of cells and arises from ATP as a byproduct in the cytosol, and its iron chelating activity is quite high enough to trap ferrous ion efficiently. Therefore, it is considered that if visible light irradiation causes the iron release from ferritin, ADP will chelate iron to produce ADP–iron complex. This complex in turn, might stimulate the oxidative stress in cells. In this study, we investigated the visible light-induced iron release from ferritin and monitored the iron release by measuring the ADP–Fe(II) complex.

Materials and methods

Materials

Horse spleen ferritin (lot number: 90K7039) was obtained from Sigma Chemical Co. (St. Louis, MO, USA), which contains *ca.* 1060 iron atoms per molecule. Desferrioxamine mesylate was purchased from Ciba Giegy (Takarazuka, Japan). 5,5-dimethyl-pyrroline-N-oxide was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). To minimize contamination by the transition metal ions, all reagents were adjusted in purified water (specific resistance of 18.0 MΩ cm; Milli-Q SP reagent water system, Millipore Co., Bedford, MA, USA) [18].

The concentration of stored iron in ferritin solution was determined by measuring ferritin iron directly in the spectrophotometer (absorbance of 1 mM ferritin iron at 480 nm = 0.263) [19]. The protein concentration of ferritin was determined by measuring ferritin protein using Bradford method [20] using the Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA, USA). The iron quantity inside ferritin used in this study was determined to be *ca.* 1060 iron atoms per molecule.

Light irradiation

Irradiation was performed with a white fluorescent light (27 W; FML27EX-N, National Co., Osaka, Japan). The irradiance delivered by this light was kept constant by adjusting the distance between the light and the reaction mixture (17,000 *lx* at the light-sample distance of 10 cm). The light output was measured with an irradiance meter (IM-3, Topcon Co., Tokyo, Japan).

UV-A irradiation was carried out with a black light (HP-6L, ATTO Co., Tokyo, Japan) emitting UV-A ranging from 320 to 400 nm with a peak emission at 365 nm.

When the irradiances of UV-A in a white fluorescent light at 17,000 *lx* and the black light were detected by a UV radiometer (UVR-3036/S2, Topcon Co., Tokyo, Japan), those were *ca.* 0.09 mW/cm^2.

Control samples were kept in darkness by covering them with an opaque black plastic sheet.

Measurement of iron released from ferritin

At various pH values, horse spleen ferritin solution (0.5 mg/ml, 400 μl) buffered with 20 mM tris-maleate with ADP was placed into a well of 24-well microplate, and irradiated for 0–60 min at 17,000 *lx* with white light or maintained in the dark at 37°C.

After the incubation, the reaction mixture was placed into the sample reservoir of a 10 kDa cut-off filter (YM-10, Millipore Co., Bedford, MA, USA), and centrifuged at 13,000 rpm for 1 h at 4°C. The supernatant was divided into two parts; one was added to a solution of desferrioxamine (1 mM final concentration), and the other was added to the corresponding volume of purified water as a control sample. After incubation for 30 min at room temperature, the absorbance of

the samples was measured with a spectrophotometer at 430 nm (U-3000, Hitachi, Ltd., Tokyo, Japan). The iron concentration of the sample was calculated by subtracting the absorbance of the control from the corresponding test sample using the molar extinction coefficient for iron ferrioxamine complex formation ($\varepsilon_{430\,nm} = 2480\,M^{-1}\,cm^{-1}$) [18,21].

Statistical analysis

All data are reported as means ± SD. Two sample comparisons were analyzed by the unpaired Student's *t*-test. The comparisons among three or more groups were analyzed by multiple comparison methods. One-way analysis of variance (ANOVA) was performed to determine the statistical significance of the data. To establish the statistical significance of the differences between pairs of means, an *a posteriori* comparison was calculated using the Tukey's test or the Dunnett's test. A correlation within each group was assessed with Pearson's correlation coefficient.

Results

Effects of pH

A solution of ferritin (0.5 mg/ml) buffered with 20 mM tris-maleate (pH 5.0 to pH 8.0) (in the absence of ADP) was incubated at 37°C for 20 min under irradiated conditions with a white fluorescent light (17,000 *lx*) or in dark (0 *lx*) (Table I). Irradiation significantly induced the iron release from ferritin at acidic pH conditions giving rise to the iron release from ferritin. Under dark conditions the irradiation did not induce a high amount of iron release. At pH 5.0, the amount of iron release from ferritin under irradiated and dark conditions were 11.1 and 5.5 μM, respectively, while those at pH 6.0 were 7.2 and 2.8 μM, respectively. The subtraction of the released iron amounts in dark samples from those in irradiated samples were 5.6 μM at pH 5.0 and 4.4 μM at pH 6.0. At neutral and basic pH conditions, the iron release was not enhanced by irradiation.

Table I. Effect of pH values on irradiation-induced release of iron from ferritin.

	Iron released from ferritin (μM)		
pH value	Dark	17,000 *lx*	Differences[#]
5.0	5.5 ± 1.5	11.1 ± 1.3**	5.6
6.0	2.8 ± 0.6	7.2 ± 0.5**,††	4.4
7.0	3.3 ± 0.7	3.5 ± 0.7†	0.2
8.0	2.9 ± 0.7	3.2 ± 0.3	0.3

A solution of ferritin (0.5 mg/ml) buffered with 20 mM tris-maleate at a different pH value from 5.0 to 8.0 was incubated at 37°C for 20 min. Values presented are arithmetic means ± S.D. of triplicates for iron concentrations detected in the medium. The significance levels between irradiated and dark conditions are expressed as **, $p < 0.01$. The significance levels between two pH conditions are expressed as †, $p < 0.05$ and ‡, $p < 0.01$. #: the differences calculated from concentrations of iron found in the columns of "Dark" and "17,000 *lx*" indicated in this Table.

Effects of ADP

Figure 1 shows that the irradiation of ferritin at pH 7.0, and 37°C for 20 min in the presence of ADP raised the iron release from ferritin in a concentration dependent fashion. The subtracted iron concentration from irradiated samples by the control sample correlated well with the ADP concentration in the range of 0.1–100 mM ($r = 0.806$).

Effects of irradiance, pH value, temperature and ferritin concentration in the presence of ADP

Figure 2 relates the pH and irradiances effects on the iron release from ferritin in the presence of 10 mM ADP. Irradiation-induced iron release from ferritin

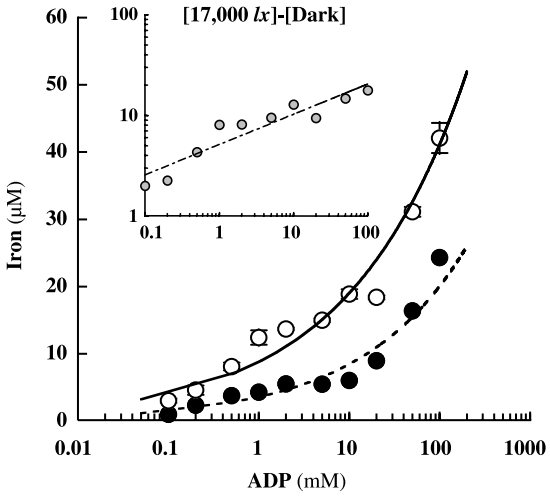

Figure 1. Effects of ADP concentration on irradiation-induced release of iron from ferritin. In the presence of ADP at various concentrations, iron release from ferritin is shown under irradiated (17,000 *lx*) (open circle) and dark conditions (closed circle). A solution of ferritin (0.5 mg/ml) buffered with 20 mM tris-maleate (pH 7.0) was incubated at graduated concentrations of ADP (0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 mM) at 37°C for 20 min. The positive correlation between the ADP concentrations and the iron release from ferritin under irradiated and dark conditions were observed ($r = 0.936$ and 0.981, respectively). The inset expresses the relationship between added ADP concentrations and net values of irradiation-induced release of iron from ferritin. The data shown are obtained by subtracting the values under dark conditions from those under irradiated conditions. Values presented are arithmetic means ± S.D. of triplicates for iron concentrations detected in the medium. The positive correlation between the ADP concentrations and the net release of iron from ferritin was observed ($r = 0.806$).

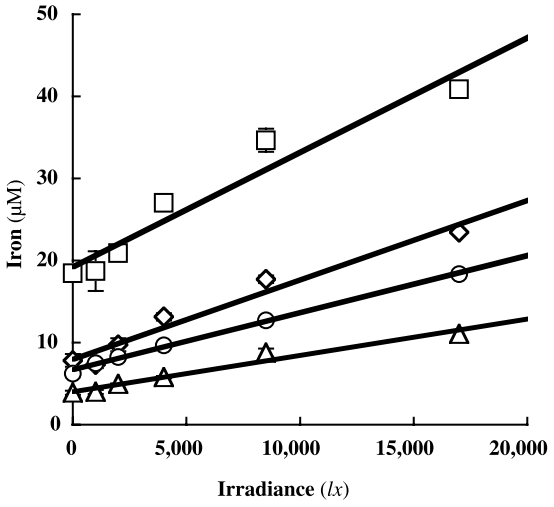

Figure 2. Effects of irradiance and pH value on irradiation-induced release of iron from ferritin in the presence of ADP. A solution of ferritin (0.5 mg/ml) buffered with 20 mM tris-maleate at a different pH value from 5.0 to 8.0 was incubated with 10 mM ADP at 37°C for 20 min. Open squares correspond to a pH 5.0; diamond, a pH 6.0; circle, a pH 7.0; triangle, a pH 8.0. Irradiances were set at 0, 1,000, 2,000, 4,000, 8,500, and 17,000 *lx*. Values presented are arithmetic means ± S.D. of triplicates for iron concentrations detected in the medium. The positive correlations between the irradiances and the iron release from ferritin were observed ($r = 0.961$, for pH 5.0; $r = 0.977$, for pH 6.0; $r = 0.999$, for pH 7.0; $r = 0.979$, for pH 8.0).

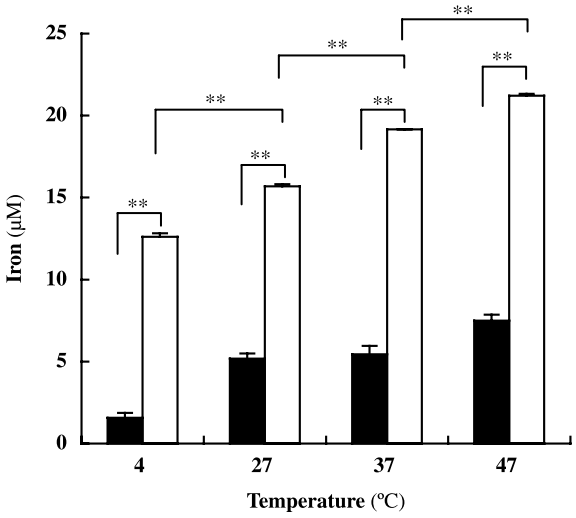

Figure 3. Effect of temperature on irradiation-induced release of iron from ferritin in the presence of ADP. A solution of ferritin (0.5 mg/ml) buffered with 20 mM tris-maleate (pH 7.0) was incubated with 10 mM ADP at 4, 27, 37, or 47°C for 20 min. Shaded columns correspond to dark conditions; open columns, under irradiated conditions (17,000 *lx*). Values presented are arithmetic means ± S.D. of triplicates for iron concentrations detected in the medium. **, $p < 0.01$.

was directly proportional to the irradiance at each pH value. The dependence of the curve in Figure 2 had a tendency to increase at lower pH values: 1.4 nM/*lx* (pH 5.0), 1.0 nM/*lx* (pH 6.0), 0.7 nM/*lx* (pH 7.0), and 0.4 nM/*lx* (pH 8.0). Also, it can be noted that concentrations of iron released from ferritin at pH 5.0 (open square) within the irradiance range of 0–17,000 *lx* were much higher (by more than 10 μM) in comparison with those at pH 6.0 (open diamond). At pH 5.0, 17,000 *lx* irradiation induced a high level of the iron release from ferritin (40.9 μM), with this value almost twice as high as that at pH 7.0 (18.3 μM) (open circle).

Next, the temperature (4, 27, 37, or 47°C) effects on the irradiation-induced iron release from ferritin were investigated. The released iron concentration differences between irradiated and dark conditions were significantly remarkable at every temperature examined (Figure 3). Because the differences of the released iron concentration from ferritin between irradiated and dark conditions were 11.0 μM (4°C), 10.5 μM (27°C), 13.8 μM (37°C), and 13.7 μM (47°C), respectively, the temperature did not influence the iron release.

The effect of ferritin concentration on irradiation-induced iron release from ferritin at pH 7.0, 17,000 *lx*, and 37°C was directly proportional to the ferritin concentration up to at least 4.0 mg/ml (data not shown).

Time dependence of iron release from ferritin

In the presence of ADP, irradiation at 17,000 *lx* remarkably induced iron release from ferritin at the release rate of 0.59 μM/min (Figure 4). This reaction proceeded by increasing the incubation time to 60 min, resulting in the detection of 35.4 μM iron released from ferritin at 60 min. On the other hand, iron was scarcely released from ferritin under dark conditions (0.11 μM/min). This result suggested the possibility that irradiation-induced iron release from ferritin was initiated by the irradiation-mediated effect of ferritin function, i.e. iron storage function, for several minutes following the onset of the irradiation. Therefore, in order to examine this possibility, we carried out an iron release experiment with discontinuous irradiation of the ferritin solution (Figure 4). When irradiation was stopped at 10 min after the start of the irradiation, the rate of iron release decreased to 0.15 μM/min. This value was almost to the same level from the previous dark conditions. After the additional irradiation was restarted at 40 min, the rate of iron release from ferritin (0.71 μM/min) returned to the previous values.

In the absence of ADP, the released iron from ferritin at pH 7.0 was not detected even under irradiated conditions for 60 min (data not shown).

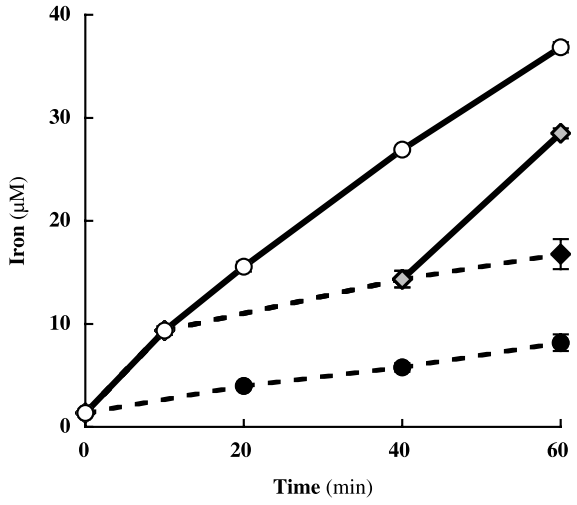

Figure 4. Time dependence of iron release from ferritin at pH 7.0 in the presence of ADP. A solution of ferritin (0.5 mg/ml) buffered with 20 mM tris-maleate (pH 7.0) was incubated with 10 mM ADP at 37°C. The solid lines with open circles show the values during continuous irradiation (17,000 *lx*). The dashed lines with closed circles show values under dark conditions. Black diamonds correspond to the amounts of iron released from ferritin in samples held in the dark after irradiation from 0 to 20 min. Gray diamonds show the amounts of iron released from ferritin in samples irradiated for 20 min after irradiation from 0 to 20 min with subsequent dark condition up to 40 min. Values presented are arithmetic means ± S.D. of triplicates for iron concentrations detected in the medium.

Effects of UV-A contained in the white fluorescent light

The white fluorescent light contains a very small amount of UV-A *(ca.* 0.09 mW/cm^2 at 365 nm, the intensity detected under irradiated conditions at 17,000 *lx*). Therefore, we examined the effect of UV-A on iron release from ferritin by using the optical filter that can cut off the wavelength range of light shorter than 390 nm. The optical filter suppressed about 39.9% of irradiation-induced iron release from ferritin (Table IIA). This result shows that approximately 60% of iron release from ferritin by the white fluorescent light was induced by the visible light.

In addition, the effect of a black light using the same energy level of UV-A *(ca.* 0.09 mW/cm^2 at 365 nm) as that of the white fluorescent light (17,000 *lx*, 20 min) on the iron release from ferritin was also examined. The concentration of released iron from ferritin by UV-A irradiation was 27.7% of the total amount of released iron concentration from ferritin by irradiation of white fluorescent light (Table IIB).

Effects of antioxidants and oxygen concentrations

Although it has been reported that $O_2^{\cdot-}$ induces Fe^{2+} release from ferritin by reducing Fe^{3+} to Fe^{2+} in the mineral core [13], irradiation-induced iron release from ferritin was not suppressed by superoxide

Table II. Effects of an optical filter, UV-A irradiation, antioxidants and oxygen concentration.

	Irradiated Conditions	Released Iron (μM)		Percentage
(A)	Dark	5.3 ± 0.3		0%
	17,000 *lx*	20.1 ± 0.6	–	100%
	+Optical filter (≤390 nm cut-off)	14.2 ± 0.2	**	60.1%
(B)	Dark	6.5 ± 1.4		0%
	17,000 *lx*	19.4 ± 0.5	–	100%
	UV-A (0.09 mW/cm^2)	10.1 ± 0.2	**	27.7%
(C)	Dark	6.6 ± 0.4		0%
	17,000 *lx*	19.4 ± 1.2	–	100%
	+SOD (1000 U/ml)	19.0 ± 0.9	N.S.	97.5%
	+Catalase (1000 U/ml)	19.0 ± 0.5	N.S.	97.5%
	+SOD/Catalase (1000/1100 U/ml)	17.7 ± 0.2	N.S.	87.4%
	+Dimethylthiourea (100 mM)	17.3 ± 0.5	N.S.	84.0%
	+Formic acid (100 mM)	19.1 ± 0.6	N.S.	98%
	+Sodium azide (100 mM)	18.4 ± 1.4	N.S.	92.7%
	+DMPO (100 mM)	19.3 ± 0.7	N.S.	99.4%
(D)	Dark	5.5 ± 0.2		0%
	17,000 *lx*	16.6 ± 0.7	–	100%
	+N_2 bubbling	15.4 ± 0.4	N.S.	89.2%
	+O_2 bubbling	14.7 ± 0.8	N.S.	82.9%

A solution of ferritin (0.5 mg/ml) buffered with 20 mM tris-maleate at pH 7.0 was incubated with 10 mM ADP at 37°C for 20 min under various conditions: (A) white light irradiation with or without an optic filter, which can cut off the wavelength range of light shorter than 390 nm; (B) UV-A irradiation at 0.09 mW/cm^2 instead of white light irradiation; (C) additions of various antioxidants; (D) pretreatment of reaction mixtures by bubbling nitrogen or oxygen gas. Values presented are arithmetic means ± S.D. of triplicates for iron concentrations detected in the medium. The inhibition rates by the indicated treatments for the irradiation-induced iron release from ferritin are expressed as percentages. The significance levels vs. each value under white light-irradiated conditions at 17,000 *lx* are expressed as **, $p < 0.01$. Abbreviation: N.S., no significance.

dismutase (SOD, 1000 U/ml) in the presence of ADP (Table IIC). Catalase (1000 U/ml), a hydrogen peroxide-scavenging enzyme, and a mixture of SOD and catalase (1000 and 1100 U/ml, respectively) also did not inhibit the irradiation-mediated iron release from ferritin (Table IIC).

In addition, the irradiation-mediated iron release from ferritin was not affected by hydroxyl radical scavengers, i.e. dimethylthiourea (100 mM) and formic acid (100 mM), a singlet oxygen quencher, i.e. sodium azide (100 mM), and a free radical scavenger, i.e. 5,5-dimethylpyroline-N-oxide (DMPO, 100 mM) (Table IIC).

Moreover, the change of oxygen concentration in the reaction media by bubbling nitrogen or oxygen gas scarcely influenced the irradiation-induced iron release from ferritin (Table IID).

Discussion

It is known that various chemical compounds and stimuli induce the iron release from ferritin via the reaction, which dissolves iron on the surface of the mineral core inside ferritin. The organic compounds chelating iron such as nitrilotriacetate [22] or the low-molecular-weight reductants small enough to pass through channels of the ferritin-constructing protein shell such as $O_2^{\cdot-}$ [13,23,24], cysteine, glutathione [10], and ascorbate [10–12] are considered to participate in the reaction. The irradiation of UV-A [14,15] or gamma-ray [16] also induces iron release from ferritin. However, irradiation with intense UV or gamma-ray causes a decline or loss of ferritin function via the destruction of its amino acid residues. On the other hand, ferritin, but not apo-ferritin, has a strong absorbance band at UV and visible regions by the existence of a mineral core, indicating that the visible light is absorbed by ferritin itself [14,15]. In order to ascertain the involvement of visible light in the iron release function, we investigated the required conditions for iron release from ferritin by visible light.

First, we investigated whether the irradiation of ferritin with a white fluorescent light induces the iron release from ferritin in the aqueous buffer solution. The irradiation at acidic pH conditions, i.e. pH 5.0 and pH 6.0, induced the marked iron release from ferritin, however, the irradiation-induced iron release from ferritin at pH 7.0 and pH 8.0 was not observed (Table I). The pH effect on the iron release from ferritin may also be related to iron uptake of ferritin, since Laulhere et al. [25] reported that the amount of iron uptake of horse spleen ferritin are higher at pH 8.4 than at pH 6.0 conditions.

The irradiation of ferritin in the presence of ADP induced iron release from ferritin even at pH 7.0 under irradiated conditions (Figure 1). ADP has no activity to reduce iron, whereas ADP can chelate with Fe^{2+} to form Fe^{2+}–ADP complex, which converts to Fe^{3+}–ADP via the oxidation using dissolved oxygen gas in the media. The possibility of Fe^{2+}–ADP to reduce Fe^{3+} inside ferritin is ruled out because the reduction potential of Fe^{3+}–ADP/Fe^{2+}–ADP ($\varepsilon^{0'} = +0.10$ V) is higher than that of Fe^{3+}-ferritin/Fe^{2+}-ferritin ($\varepsilon^{0'} = -0.19$ V) [26]. This is consistent with the observation that ADP does not induce the distinct iron release from ferritin under dark conditions (Figure 4). The lower, but significantly high amount of iron release in the presence of ADP under dark conditions was observed in the concentration- and time-dependent sections of our experiment (Figures 1 and 4). This can be explained by the process of inhibition of iron re-uptake by ferritin. Namely, although ferritin physiologically releases both iron to cytoplasm to meet the demand from cells and takes up an excess of iron by ferritin itself, the strong iron chelation by ADP inhibits iron uptake resulting in the increased iron release even under dark conditions.

We further investigated the possibility that irradiation-induced iron release from ferritin is affected by several environmental factors such as temperature, oxygen, antioxidants, and on/off light switching.

(i) *Temperature effects*: The temperature increases from 37 to 47°C under irradiated conditions enhanced the iron release from ferritin *ca.* 2.0 μM (Figure 3), but this value is quite small. The temperature of the sample solution did not rise by white light irradiation at 17,000 *lx* for 20 min. The temperature rise under irradiated conditions influences the noted differences of the released iron concentration a little if it influences.

(ii) *Oxygen (reactive oxygen species) and antioxidants effects*: The exclusion of oxygen in the reaction media did not influence the iron release by visible light in the presence of ADP (Table IID), suggesting that the irradiation-induced iron release from ferritin occurs even under anaerobic conditions. This implies that oxygen gas (or oxygen gas derivatives) might not be involved in irradiation-induced iron release from ferritin. Further, the addition of the antioxidant enzyme such as SOD, catalase, and small molecular antioxidants such as dimethylthiourea, formic acid, sodium azide and DMPO, did not influence the iron release. SOD and catalase are not likely to be small enough to get in the ferritin shell, however, they can scavenge $O_2^{\cdot-}$ and H_2O_2 outside the ferritin shell. If $O_2^{\cdot-}$ and/or H_2O_2 may participate in the iron release from

ferritin, iron release from ferritin might be affected by the addition of enough amount of SOD and catalase. But, this is not the case. The experimental results on Table IIC showed that the found addition of SOD and catalase did not show any effect on the iron release from ferritin. Further, bubbling of nitrogen and oxygen gas did not affect the iron release from ferritin (Table IID). Therefore, there may be neither the possibility of generation of both oxygen species outside the shell nor the influence of them on a mineral core inside a protein shell under irradiated conditions. All these results clearly demonstrate that reactive oxygen species such as $O_2^{\cdot -}$, H_2O_2, $^{\cdot}OH$, $^1O_2$, and $CO_2^{\cdot -}$, do not play an important role in the iron release from ferritin (Table IIC).

(iii) *On/off light switching effects*: The irradiation-induced iron release was immediately suppressed by turning off the light, and then the iron release was restarted by turning on light (Figure 4). If the light irradiation resulted in protein damage, the iron release from ferritin would not be suppressed by turning off the light. Therefore, the irradiation did not inflict critical damage to protein structures and iron storage functionality of ferritin. Based on these facts, the irradiation-induced iron release from ferritin in the presence of ADP may occur without the oxidative damages of ferritin by reactive oxygen species generated by photosensitization.

As for the mechanisms for the irradiation-induced iron release from the mineral core, Aubailly et al. [14] reported two photochemical mechanisms for the irradiation-induced iron release from the mineral core inside ferritin as shown below.

$$\mathrm{FeO{-}OOCR} + \mathrm{H_2O} \xrightarrow{h\nu} \mathrm{Fe^{2+}} + \mathrm{RCOO^{\cdot}} + 2\,{}^{-}\mathrm{OH} \quad \text{(a)}$$

(R: glutamic acid residue)

$$\mathrm{Fe(III)OOH} + \mathrm{H_2O} \xrightarrow{h\nu} \mathrm{Fe^{2+}} + 2\,{}^{-}\mathrm{OH} + {}^{\cdot}\mathrm{OH} \quad \text{(b)}$$

Both mechanisms involve the reactive oxygen radical species in the reaction course, which did not occur in our case. As discussed above, the irradiation-induced iron release from ferritin was hardly influenced by various free radical scavengers. Therefore, it is our belief that this irradiation-induced iron release from ferritin mainly takes place by light absorption of the mineral core inside ferritin. There are several reports that the Fe^{3+}-chelates with some chelating agents were decomposed only by the exposure to sunlight accompanying the photoreduction of Fe^{3+} to Fe^{2+} [27,28]. Similar mechanism might take place in our reaction conditions employed.

The iron release from ferritin under irradiated conditions was observed, especially in the presence of millimolar order of ADP. Such a high ADP concentration may be considered to be relatively high to be referred as the physiological condition. However, intracellular ADP concentration was known to be at the level of 0.05–2 mM [29]. This means that ADP concentration is enough to induce iron release from ferritin at the intracellular level. As for the pH conditions, it is known that the differentiated keratinocytes in the corneal layer of human epidermis are acidic pH conditions ranging from pH 3.0 to pH 6.5 [30]. So, our experimental findings are applied at least in some physiological conditions described above.

In this paper, we have shown for the first time that visible light can induce iron release from ferritin by direct photoreduction of ferric ion on the mineral core surface. However, the precise reaction mechanism for the photoreduction at the molecular level is a future challenging problem now in progress in our laboratory.

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