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A soluble-form of pro-oxidant lumazine isolated from cyanobacterial cells generates superoxide anion under near-UV irradiation

Hitoshi Shibata *, Tetsuji Noda, Yasushi Ogura, Katsuaki Suginaka, Yoshihisa Matsui, Yoshihisa Ozoe, Yoshihiro Sawa, Yasuhisa Kono

Department of Life Science and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue, Shimane 690, Japan

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Abstract

By monitoring the generation of superoxide anion radical (O_2^-), a new and water-soluble near-UV photosensitizable compound was isolated from filamentous cyanobacterium *Phormidium lapideum*. This compound, having an absorption peak at 325 nm at pH 7.0, was identified as lumazine (2,4(1*H*,3*H*)-pteridinedione) on the basis of absorption, fluorescence, MS and NMR spectra. Lumazine was also detected in the extract from unicellular cyanobacterium, *Synechococcus* PCC 7942. In a lumazine-sensitized reaction, NO_2^- accumulation from the reaction of NH_2OH with O_2^-, proceeded linearly, with the time of irradiation. The accumulation was inhibited by superoxide dismutase. O_2-Consumption in the presence of NH_2OH was accompanied by a formation of half-molar NO_2^-. Moreover added cytochrome *c* apparently abolished the observed oxygen consumption, indicating that the excited lumazine generated O_2^- from O_2 stoichiometrically. These results indicate that near-UV photons reaching into the cyanobacterial cells cause oxidative stress by producing active species of oxygen via the sensitized reaction of pro-oxidant(s) such as lumazine.

Keywords: Active oxygen; Cyanobacterium; Lumazine; Near-UV; Pro-oxidant

1. Introduction

There is increasing concern that near-UV light (290–400 nm) may impinge on the earth's surface as a results of depletion of ozone layer in the atmosphere. Irradiation by near-UV especially by UV-B which is defined as 280–315 nm, but often expanded to 320 nm [1], is one of the most common environmental sources of stress to most organisms. Because there are multiple responses to near-UV, it is difficult to follow a single set of events (see review Ref. [2]); much depends on the initial photoreceptor and subsequent reactions. One representative near-UV photoreceptor is DNA; near-UV photons induce photo-damage to DNA, then trigger lethal and multigenic effects. Beside the direct reaction to DNA, the observation of oxygen enhancement of lethality for near-UV is strong evidence that perhaps more than 90% of lethal events are the results of photosensitizing reactions of the photodynamic type, involving pro-oxidant working as the endogenous photosensitizers. The rare thiolated tRNA molecules have been identified to be one of photosensitizers involved in the lethal events caused by near-UV in *Escherichia coli* [3] and *Salmonella typhimurium* [4]. Cellular components absorbing near-UV light, such as porphyrin derivatives, riboflavin, pyridoxal phosphate, NAD(P)H, some amino acids and polypeptides are reported to generate active species of oxygen under near-UV irradiation [2]. In other respects related to the near-UV photosensitizers, plastoquinone is known as the near-UV sensitizer mediating degradation of the 32-kDa photosystem II reaction center protein in the UV spectral region [5]. Photoreactivating enzyme (photo-lyase, EC 4.1.99.3, monomerizes pyrimidine dimers in UV-irradiated DNA) of cyanobacterium, *Anacystis nidulans*, contains 7,8-didemethyl-8-hydroxy-5-deazaribofalvin and reduced FAD as the intrinsic chromophores in the near-UV region [6,7]. There are two other reports indicating the presence of compounds absorbing near-UV light in cyanobacterial cells. The UV-absorbing substance having an absorption peak near 320 nm is detected in cyanobacterial cells, floating on the sea surface in the Great Barrier Reef [8]. Another UV-A/B absorbing pigment with absorption maxima at 312 and 330 nm has been isolated from the

* Corresponding author. Fax: +81 852 326598.

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terrestrial filamentous cyanobacterium *Nostoc commune* [9].

In previous studies, we detected shock protein synthesis under near-UV irradiation in cyanobacterial cells, *Synechoccocus* PCC 7942 [10]. One of the most inducible proteins was identified as the GroEL translation product [11] which is the heat shock protein. Without irradiation, H_2O_2-induced proteins in cyanobacterial cells overlapped many near-UV shock proteins as the case of *Escherichia coli* [12,13], *S. typhimurium* [13], and *Drosophila* cells [14]. These results suggest that near-UV irradiation generates active species of oxygen such as O_2^- and H_2O_2 in cyanobacterial cells. O_2^--generation monitored by the cytochrome (Cyt) *c* reduction which was inhibited by superoxide dismutase (SOD) was observed under near-UV irradiation to the whole cells [11] or to the low-molecular-weight (*low-mol-wt*) fraction prepared by gel-filtration on Sephadex G-25, of the extract from *Synechococcus* PCC 7942 cells [10]. These results suggested that cyanobacterial cells contain the low molecular weight near-UV pro-oxidant(s) which absorbs near-UV photons, and is easily extractable with water.

We have isolated the near-UV sensitizable pro-oxidant that generates O_2^- under near-UV irradiation from filamentous cyanobacterium *Phormidium lapideum* and identified it to be lumazine (2,4(1*H*,3*H*)-pteridinedione) by absorption, fluorescence, MS and NMR spectra. HPLC analysis, spectrophotometric and near-UV sensitizable properties confirmed that this pro-oxidant is also present in unicellular *Synechococcus* PCC 7942 cells.

2. Materials and methods

2.1. Cyanobacterial cells

Phormidium lapideum and *Synechococcus* PCC 7942 cells were cultured as described previously in Refs. [15] and [10], respectively.

2.2. Materials

Cyt *c*, and authentic lumazine, pterin and folic acid were the products of Sigma, and Tokyo Kasei, Tokyo, respectively. CuZn-SOD was the generous gift from Toyo Jozo Co., Shizuoka, Japan. Other reagents were analytical grade and purchased from Wako Chemical Industry, Osaka, Japan. UV-D33S pass-filter was obtained from Toshiba Glass Co. Ltd., Tokyo.

2.3. Isolation of near-UV photosensitizable compound

Frozen *P. lapideum* cells (ca. 100 g wet weight) were suspended in 100 ml MeOH then passed two times through a French press (1100 p.s.i.). The homogenate was centrifuged at 15 000 × *g* for 15 min, then its volume of the supernatant was reduced under vacuum to about 30 ml. After chromatography on Sephadex G-25 (30 cm × 5 cm i.d.), equilibrated and eluted with distilled H_2O, pooled fraction containing *low-mol-wt* compounds was concentrated to about 200 μl (this was designated herein *low-mol-wt* fraction), then applied to preparative TLC (Kieselgel 60 GF_{254}, Merck). The plates were developed in the upper layer of *n*-BuOH/AcOH/H_2O (4:1:5). In some cases the second solvent system (*n*-BuOH/NH_4OH = 5:1) was used. Fluorescent band (R_f = 0.41) after the one-dimensional separation was scraped and extracted with 50% MeOH, then concentrated to an appropriate volume. The sample was further purified by HPLC on ODS-80$_{TM}$ column (4.6 × 250 mm, Tosoh, Japan) using the solvent system (4% MeOH, 0.2% AcOH in H_2O) at a flow rate, 1.0 ml/min. The absorbance at 320 nm was monitored. The photosensitizable compound was eluted at 11.5 min as a symmetrical peak and pooled; the yield was ca 100 μg from 100 g (wet weight) cyanobacterial cells.

2.4. Irradiation of near-UV to generate O_2^-

Near-UV fluence (295–390 nm; 400 μm w/cm^2) was obtained by filtering light with UV-D33S filter from a 200-W high pressure mercury arc enclosed in a glass outer envelop equipped with a Pyrex glass filter and running water cooling system, as described previously in Ref. [10]. Monochromatic light was supplied from an Okazaki Large Spectrograph [16], to obtain action spectra for near-UV induced O_2^--generation, according to the procedures described in Ref. [17].

2.5. Detection and estimation of generated O_2^-

Cyt *c* reduction [18] was monitored during purification of the sensitizable compound. In other cases, O_2^--generation was estimated by NO_2^--accumulation from the reaction of NH_2OH with O_2^-, in a reaction mixture described in Ref. [19].

2.6. Analytical methods

Electron impact mass (EIMS) spectra were obtained at 70 eV with a Hitachi M-80B/M-0101 mass spectrometer. ^{13}C- and ^{1}H-nuclear magnetic resonance (NMR) spectra were measured at 100 and 400 MHz, respectively, with a JOEL JNM-A400 spectrometer. O_2-consumption was monitored with an oxygen electrode system (Rank Brothers, Cambridge, UK). Absorption and fluorometric spectra were obtained with Beckman DU650 spectrophotometer and Hitachi 650-10S spectrofluorometer, respectively.

3. Results

We reported [10] the occurrence of near-UV photosensitizer(s) in the *low-mol-wt* fraction from *Synechococcus*

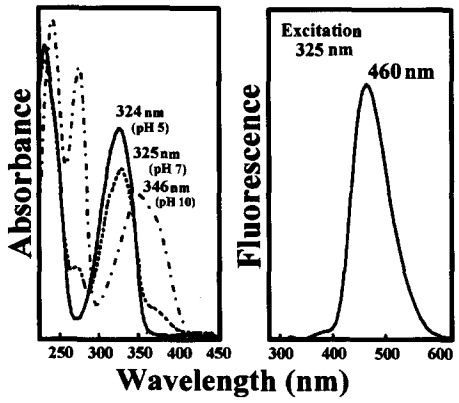

Fig. 1. Spectrophotometric (left) and fluorometric emission (right) spectra of the near-UV sensitizable compound isolated from cyanobacterium, *P. lapideum*. The wavelengths of each peak, and the pH of solution were indicated.

PCC 7942. During the near-UV irradiation to the fraction, reduction of Cyt *c* was observed and it was inhibited by SOD, indicating the generation of O_2^- via the sensitized near-UV absorbing compound(s). In a preliminary experiment, at least 10 fluorescent compounds were separated from the *low-mol-wt* fraction on a two-dimensional TLC analysis. The extracted two compounds from the plate generated O_2^- under near-UV irradiation, but others did not. Here we obtained ca. 500 μg of one near-UV sensitizable compound from 500 g (fresh weight) *P. lapideum* cells by the repeated extraction and isolation procedures. Fig. 1 shows the absorption and fluorescence characteristics of the purified compound. At pH 5.0, the absorption spectrum of the compound had maxima at 228 and 324 nm, while at pH 10.0, the spectrum exhibited three peaks at 234, 270 and 346 nm. The fluorescence property of the compound at pH 7.0 showed an emission maximum at 460 nm when excited at 325 nm.

The structure of the near-UV sensitizable compound was confirmed as lumazine (2,4(1*H*,3*H*)-pteridinedione) by comparison of its spectral data with those of authentic lumazine. In EIMS analysis (Fig. 2), the sensitizable compound gave a molecular ion peak (*m/z* 164) and three major fragments at *m/z*, 121 (M^+-CONH), 93 (M^+-CONHCO), and 66 (M^+-CONHCONHC). The sensitizable compound and lumazine showed only two main peaks in ^{13}C- and ^1H-NMR spectra, which were assigned to be 6- and 7-carbons of pteridine skeleton and hydrogens bound to them (Table 1). ^{13}C-peaks of carbons other than 6- and 7-carbons were hardly observed because of low concentrations. COSY (correlated spectroscopy) methods for two-dimensional ^1H-NMR revealed that the two protons were adjacent to each other ($J = 2.44$ Hz). Several weak peaks that were not observed in the case of lumazine were detected, suggesting a contamination of a little amount of probable pyrimidine derivative(s) in the purified preparation (data not shown). Authentic lumazine showed identical spectrophotometric properties with those shown in Fig. 1, and also worked as the near-UV sensitizer (see later).

Table 1
^{13}C- and ^1H-NMR data of the near-UV sensitizable compound purified from *P. lapideum* and of the authentic lumazine

Compound	$\delta^{13}C$ (D_2O)	δ^1H (D_2O)
	C-6 or C-7	H-6 or H-7
Near-UV sensitizable compound	143.8 or 152.3	8.54 or 8.80
Lumazine	143.7 or 152.8	8.59 or 8.71

The *low-mol-wt* fraction prepared from unicellular *Synechoccocus* PCC 7942 cells was conducted to preparative TLC separation, then the fluorescent spot was analyzed by HPLC (Fig. 3). The main symmetrical elution peak was accompanied with a small peak (Fig. 3A). This small peak was also detected in the extract from *P. lapideum* (Fig. 3B). The compound in the main peak shown in Fig. 3A was further confirmed to be lumazine by the absorption spectra, EIMS data (data not shown), and near-UV sensitizable properties. From these results, we concluded that filamentous (*P. lapideum*) and unicellular (*Synechococcus* PCC 7942) cyanobacterial cells contain lumazine which is extractable in the *low-mol-wt* fraction.

Near-UV irradiation to a solution containing lumazine

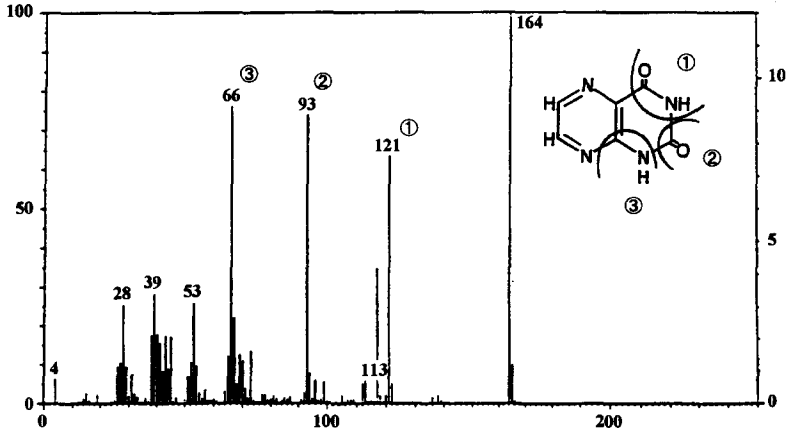

Fig. 2. Mass spectrum (EIMS) of the isolated near-UV sensitizable compound from *P. lapideum*. The inset shows the structure of lumazine and the possible fragmentations detected in the spectrum.

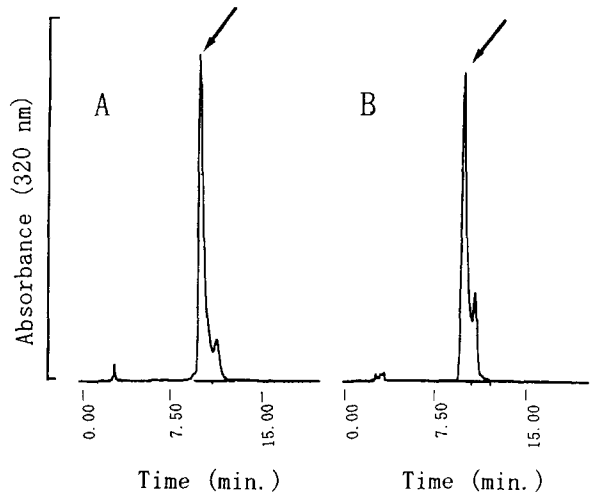

Fig. 3. HPLC analysis of the near-UV sensitizable compound in the *low-mol-wt* fractions prepared from *P. lapideum* (A) and *Synechococcus* PCC 7942 (B).

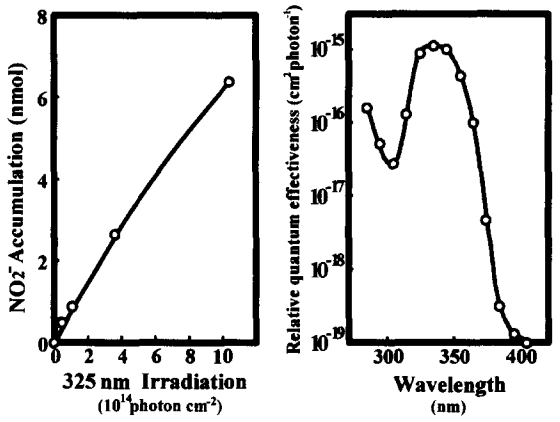

Fig. 5. Photon-dependent (left) and action spectra (right) of NO_2^--accumulation in the near-UV sensitized reactions of lumazine. The reaction mixture contained 13 mM KH_2PO_4, 35 mM $Na_2B_4O_7$, 0.1 mM EDTA (pH 7.8), 2 mM NH_2OH and 58.8 μM lumazine (OD of 0.5 at 324 nm). After near-UV irradiation for 30 min, the amounts of NO_2^- were determined.

reduced Cyt *c*, and the inhibition of the reduction by the added SOD was observed. To characterize the near-UV sensitized reaction of lumazine, O_2-consumption (Fig. 4, left), or NO_2^--formation from the reaction of NH_2OH with the generated O_2^- (Fig. 4, right) were followed in certain reaction mixtures. In a mixture containing 280 μM lumazine, O_2-consumption proceeded linearly at least for 10 min of irradiation (line 1) with a rate, 7.2 $\pm$ 0.3 nmol/min. Although added SOD (50 nM) to the mixture had no effect on the rate of the O_2-consumption (line 3), Cyt *c* (1.2 mg/ml) apparently abolished the O_2-consumption (line 2). Hydroxylamine (2 mM) stimulated the O_2-consumption

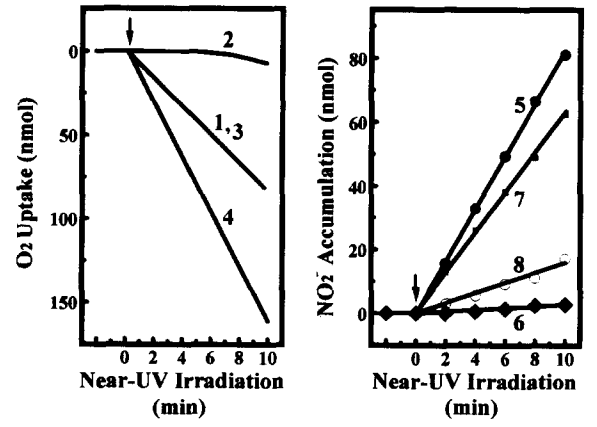

Fig. 4. Oxygen consumption (left) and O_2^--generation assayed by NO_2^--accumulation (right) during the lumazine-sensitized reaction. The basal reaction mixture (4 ml) contained 50 mM K-phosphate (pH 7.8) and 280 μM lumazine (line 1). To the mixture, 1.2 mg/ml Cyt *c* (line 2), 50 nM SOD (line 3), or 2 mM NH_2OH (line 4) was added. For the determination of accumulated NO_2^- by the near-UV sensitized reaction of pteridine compounds (280 μM) in the presence of 2 mM NH_2OH, at the 2-min of intervals of irradiation an aliquot was taken for the colorimetric assays. Line 5, lumazine; line 6, lumazine + 50 nM SOD; line 7, pterin; and line 8, folic acid. Arrows indicated the start of near-UV irradiation.

with a rate 15.0 $\pm$ 1.1 nmol/min (line 4), and thus consumption of 150 nmol O_2 within a 10-min irradiation was observed. In another assay of O_2^--generation, the accumulation of NO_2^- (Fig. 4 right, line 5), near-UV irradiation to lumazine accumulated NO_2^- almost time-dependent manner and after 10-min of irradiation, 78 $\pm$ 5.1 nmol NO_2^- were estimated. The accumulation of NO_2^- was inhibited completely by SOD (line 6). The derivatives of lumazine having pteridine skeleton, pterin (line 7) and folic acid (line 8) at the same concentration of lumazine worked as pro-oxidant with lower efficiency for the O_2^--generation than that of lumazine.

On the other hand, O_2^--generation in the lumazine-sensitized reaction, assayed by the accumulation of NO_2^-, was linearly dependent on the intensity of 325 nm of monochromatic light up to 10×10^{14} photon cm^{-2} (Fig. 5, left), which was supplied from Large Spectrograph [15] of the National Institute for Basic Biology. Action spectrum for O_2^--generation (Fig. 5, right) by the lumazine-sensitized reaction in the near-UV region showed a relatively broad peak, but centered around 320–340 nm. The *low-mol-wt* fraction-sensitized reaction, however, showed a non-linear relationship on the intensity of irradiated photons of 325 nm and its action spectrum was broader than that of lumazine (data not shown).

4. Discussion

Near-UV irradiation to the suspension of intact cyanobacterial cells [11] or to the extracted *low-mol-wt* fraction [10] caused generation of O_2^-. We have confirmed that one near-UV pro-oxidant in *P. lapideum* is lumazine by the spectrophotometric and spectrofluorometric properties (Fig. 1), EIMS (Fig. 2) and NMR (Table 1) data. Lumazine was also detected in the extract from *Synechococcus* PCC 7942

cells (Fig. 3). The occurrence and bioluminescent properties of the lumazine-derivative, 6,7-dimethyl-8-ribityl-lumazine (DR-lumazine) as the bound prosthetic group to the lumazine protein prepared from bioluminescent bacterium (*Photobacterium phosphoreum*) has been characterized [20,21]. There is no report yet on the lumazine protein originated from cyanobacteria. Spectrophotometric properties of the UV-A/B absorbing pigment detected from *Nostoc commune* [9] are different from those of lumazine. Thus this report may be the first identification of lumazine from cyanobacterial cells; however, the near-UV-absorbing substance [8] from cyanobacterium floating on the sea surface may be lumazine because absorption spectrum in an aqueous solution [8] is similar to the peak observed at pH 7 as shown in Fig. 1.

According to the mechanism of O_2^--generation in the near-UV sensitized reaction [22] and the scheme of photo-oxygenation leading to O_2^--generation [23], near-UV photons will convert lumazine (L) to triplet form ^3L

$$L + h\nu \rightarrow {}^3L \quad (1)$$

then, ^3L will react either with O_2 generating O_2^- or with a substrate producing the one-electron reduced form of lumazine (L^-) which reduces O_2 resulting in the generation of O_2^-. Lumazine is known as a good substrate for xanthine oxidase (XO) [24,25], and reduces XO under certain conditions (high pO_2 and low substrate concentration) to the partially reduced form of XO which brings about the univalent reduction of O_2 [25]. According to these properties, lumazine itself may serve as the substrate to donate one electron to the ^3L in the reaction of the photo-oxygenation.

$$^3L + O_2 \rightarrow L_{OX} + O_2^- \quad (2)$$

or

$$^3L + L \rightarrow L^- + L_{OX} \quad L^- + O_2 \rightarrow L + O_2^- \quad (3)$$

The sum of two reactions shown in Eq. (3) is equal to Eq. (2). The fate of L_{OX} is not clear yet; however, a slight but significant decrease of the absorption spectrum of lumazine was observed (data not shown) after the sensitized reaction, suggesting that a small portion of lumazine is destroyed under near-UV irradiation.

The observed O_2-consumption (Fig. 4, line 1) may imply the balance of dissolved O_2 between the consumption by Eq. (2) or Eq. (3) and the O_2-evolution by the following equations,

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (4)$$

that is, the sum of $2 \times (2) + (4)$ is

$$2 \times {}^3L + O_2 + 2H^+ \rightarrow 2 \times L_{OX} + H_2O_2 \quad (5)$$

SOD catalyzing or accelerating reaction in Eq. (4) had no effect on the apparent O_2-consumption in the sensitized reaction of lumazine (line 3). Thus, the observed O_2-consumption (Fig. 4, line 1) would be the overall reactions shown by the reactions in Eq. (1), Eq. (2) or Eq. (3) and spontaneous dismutation reaction in Eq. (4).

Cyt *c* react with O_2^-,

$$\text{Cyt } c(Fe^{3+}) + O_2^- \rightarrow \text{Cyt } c(Fe^{2+}) + O_2 \quad (6)$$

thus, the observation that added Cyt *c* apparently abolished the O_2-consumption (line 2), reflecting the reactions in Eq. (2) or Eq. (3), and Eq. (6).

The 2-fold faster O_2-consumption observed in the presence of NH_2OH (Fig. 5, line 4) may be explained by the equation [19].

$$NH_2OH + 2O_2^- + H^+ \rightarrow NO_2^- + H_2O_2 + H_2O \quad (7)$$

From these reaction mechanism, we concluded that near-UV sensitized-lumazine generate only O_2^-. Some near-UV sensitizers generate O_2^- with concomitant generation of singlet oxygen [1], although only O_2^--generation was observed from NAD(P)H by near-UV irradiation [15]. According to Eq. (2) or Eqs. (3 NO TRANSLATION 4), and as H_2O_2 reacts with metal ions to produce a strong oxidant, hydroxyl radical, which is readily reacts with numerous cellular components, near-UV will be a cause of oxidative stress in cyanobacterial cells. We already reported that near-UV irradiation to cyanobacterial cells induced the synthesis shock proteins, many of which were also induced by the added H_2O_2 [10,11].

O_2^--generation by lumazine-sensitized reaction was linearly dependent on the intensity of 325 nm of monochromatic light (Fig. 5, left); however, the generation by the *low-mol-wt* fraction showed a nonlinear relationship. Action spectrum for O_2^--generation (Fig. 5, right) by lumazine has a broad peak, but centered around 320–340 nm. The *low-mol-wt* fraction showed the almost same efficiency for O_2^--generation by photons of 300–370 nm. These results may indicate that other near-UV photosensitizable compound(s) than lumazine such as porphyrin derivatives, riboflavin, pyridoxal phosphate, NAD(P)H, some amino acids, polypeptids [2], pterin and folic acid working as the water-soluble near-UV sensitizer [2], as well as near-UV absorbing pigment(s) are extracted in the *low-mol-wt* fraction from cyanobacterial cells. We have isolated from the *low-mol-wt* fraction, another sensitizable compound (mol wt, 220; absorption maxima at 273 and 324 nm at pH 7.0), which also reduce Cyt *c* under near-UV irradiation and the reduction was inhibited by SOD, but its molecular structure is not yet elucidated.

To intercept physiologically damaging wavelength of environmental radiation, flavonoids in epidermal cells of higher plants [26], mycosporine-like amino acids in marine organisms [27], and UV-A/B protecting pigment in cyanobacterium *Nostoc commune* [9] are reported to play a role by filtering near-UV between 310–360 nm. Taken together, the non-linear relationship between the O_2^--generation and intensity of irradiation, and a broad action spectrum, observed in the cases of the *low-mol-wt* fraction may reflect the total response of the extracted compounds to near-UV photons.

Lumazine is a catabolic product from folic acid or biopterin via pterin. Pterin deaminase (EC, 3.5.4.11), converting pterin to lumazine, has been purified and characterized from *Bacillus megaterium* [28]. DR-lumazine (6,7-dimethyl-8-ribityl lumazine) is a precursor of riboflavin, and lumazine could be a product of photo-degradation of DR-lumazine metabolite, as luminochrome is a product from photo-degradation of flavin [29]. The fact that the catabolite of important components for cellular functions is the cause of oxidative stress may need occasion no surprise.

Acknowledgements

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