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Mechanistic Study and the Influence of Oxygen on the Photosensitized Transformations of Microcystins (Cyanotoxins)

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Abstract

Microcystins (MCs) produced by cyanobacteria are strong hepatotoxins and classified as possible carcinogens. MCs pose a considerable threat to consumers of tainted drinking and surface waters, but the photochemical fate of dissolved MCs in the environment has received limited attention. MCs are released into the environment upon cell lysis along with photoactive pigments including phycocyanin and chlorophyll *a*. The concentrations of MCs and pigments are expected to be greatest during a bloom event. These blooms occur in sunlit surface water and thus MCs can undergo a variety of solar initiated or photosensitized transformations. We report herein the role of oxygen, sensitizer, and light on the photochemical fate of MCs. The phycocyanin photosensitized transformation of MCs is elucidated, and photosensitized isomerization plays an important role in the process. The UV-A portion of sunlight was simulated using 350 nm light and the phototransformations of three MC variants (-LR, -RR, -LF) were investigated. Singlet oxygen leads to photooxidation of phycocyanin, the predominant pigment of cyanobacteria, hence, reducing the phototransformation rate of MCs. The phototransformation rate of MC-LR increases as pH decreases. The pH effect may be the result of MCs association with phycocyanin. Our results indicate photosensitized processes may play a key role in the photochemical transformation of MCs in the natural water.

Introduction

Cyanobacteria produce lethal toxins and pose a risk to human health. Among the most common types of cyanotoxins found in water are microcystins MCs (1). The increasing eutrophication of fresh and brackish water by human activity has increased the occurrence and intensity of cyanobacterial blooms. MCs are monocyclic heptapeptides and the key component for their biological activity is linked to a unique nonpolar amino acid, 3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid, also known as Adda (2). Epidemiological data indicate high rates of liver cancer in China due to long-term exposure to trace amounts of cyanotoxins in water (3). Concern regarding the hepatotoxicity of MCs prompted the World Health Organization (WHO) to adopt a provisional limit for microcystin-LR (MC-LR), the most common MC congener, of 1.0 $\mu\text{g/L}$ in drinking water (4).

MCs are primarily confined within in the active cyanobacteria cell and released upon lysis of the cell. Under natural circumstances, relatively high extracellular MCs concentrations ($\mu\text{g/L}$ to mg/L) appear during the breakdown of a cyanobacterial bloom (5). MCs are stable

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Supporting Information Available

The procedure of synthesis and identification of 6 (Z) Adda MC-LR; Figures showing UV and MS spectrums of 6 (Z) Adda MC-LR; Absorbance spectrum of phycocyanin; UV lamps and solar spectrums. This material is available free of charge via the Internet at <http://pubs.acs.org>.

compounds and relatively persistent in the aquatic environment (6). The environmental fate of dissolved MCs is not well characterized. While bacterial degradation of MCs can occur, in general, there is a lag period of several days or weeks (7). While UV light near the absorption maximum of the MCs (238 nm) can lead to the degradation of MCs, solar irradiation in the absence of other substrates has a minimal effect on the fate and decomposition of MCs (8,9). However, in the presence of pigments or humic substances (HS), photosensitized reactions appear to play an important role in the environmental transformation of MCs and may be applicable to specific water treatment (10–13).

Cyanobacteria are known to produce photosynthetic pigments including phycocyanin and chlorophyll *a*. Phycocyanins, the accessory pigment characteristic to cyanobacteria, accounts for up to 20% of the protein in cyanobacteria. Phycocyanin is a blue, water-soluble, and strong light absorbing pigment which is released with MCs upon cell lysis. Under bloom conditions, phycocyanin can be used as an indicator of cyanobacterial blooms (14).

Upon absorption of light, phycocyanin is excited to a singlet state, which rapidly intersystem crosses to the lower energy triplet state, eq 1. Phycocyanin³ can undergo direct electron or energy transfer with a variety of substrates (Subs), eq 2 (15). Phycocyanin³ can also lead to the formation of reactive oxygen species (ROS) by energy or electron transfer with molecular oxygen, eq 2. Competition between the reactions involving phycocyanin³ depends on the structure of the substrate and the concentrations of phycocyanin, substrate, and oxygen. Both eqs 2 and 3 can lead to photooxidation. Type I photooxidation occurs when the substrate $\cdot$, $\cdot^-$, or $\cdot^+$ reacts with molecular oxygen, eq 4, whereas type II photooxidation is typically associated with the formation and subsequent reactions of singlet oxygen with the substrate, eq 5.

Gajdek et al. (16) and Robertson et al. (12) suggested that phycocyanin generated both singlet oxygen and superoxide anion radical in the presence of molecular oxygen and sunlight; however, the roles of singlet oxygen, superoxide anion radical, and type I and II photooxidation have not been distinguished. Tsuji et al. observed minimal degradation of MC by exposure to sunlight alone, but the addition of pigments extracted from cyanobacteria accelerated their decompositions (11). A number of authors proposed that direct photosensitized transformation of MCs is important in their environmental fate (10–13,16), but the mechanistic understanding of the phototransformation of MCs is incomplete. The primary aim of our study is to characterize the roles of the photosensitized transformation and photooxidations (type I and II) of MCs under environmentally relevant conditions.

Experimental Section

Materials

MC-LR was purified from a laboratory culture of *Microcystis aeruginosa* (CCMP299) using the procedure described previously (17). MC-RR and MC-LF were purchased from ALEXIS.

NaN₃ and D₂O were purchased from Sigma-Aldrich. Molecular weight cut off filters were purchased from Fisher Scientific.

Isolation of Phycocyanin

Phycocyanin was also isolated from *Microcystis* CCMP299 obtained from NIEHS Toxic Algae Culture Center at Florida International University. Isolation of phycocyanin was achieved following the procedure reported by Gajdek et al. (16). The harvested cells were suspended in water and treated with an ultrasonic cell disrupter four times for 30 s. After cell disruption, the solution was centrifuged and filtered through GF/C discs (Whatman). The supernatant was passed through 10 g C-18 sep-pack cartridges (Fisher Scientific) to purify the phycocyanin, yielding a blue solution. The eluate was evaporated to dryness at 40 °C using speed Vac (SPD 111, Savant). The amount of isolated phycocyanin was measured gravimetrically. Solutions were prepared by transferring a measured weight of phycocyanin in a specific volume of distilled water. The absorption spectra of the isolated phycocyanin is shown in Figure 1S, Supporting Information. The measured $\epsilon_{618\text{ nm}}$ of the isolated phycocyanin is in agreement with reported by Robertson et. al (12).

UV Irradiation

A Rayonet photo reactor was used to simulate the UV and visible portions of the solar spectrum. Lamps with emissions centered at 350 nm (350 ± 50 nm) or 450 nm (400 ± 50 nm) were employed. A xenon lamp (Oriel) with a 500 nm cut off filter was used for longer wavelength experiments. The emission profiles and densities were measured using an Irrad 2000 Spectradiator (Ocean Optics, Inc) in the reaction vessels and under the experimental conditions, shown in Figure 2S, Supporting Information. Eighty mL pyrex test tubes with i.d. 2.5 cm were employed for the irradiation experiments. Samples, 1.0 mL, were taken for analysis at various time intervals. The reaction solution was kept at 32 ± 1 °C.

Analysis

The concentration of MCs was monitored by HPLC with photodiode array detector (Beckman 166 detector). Separations were performed on a Prosphere ODS column (250 mm × 4.6 mm I.D.; 5 µm particle size). The mobile phase consisted of 60% CH₃OH and 40% 20 mM ammonium acetate aqueous buffer solution. Chromatograms were analyzed and integrated at 238 nm. The coefficient of variation was <2% for triplicate HPLC analysis.

MC-LR Association with Phycocyanin

Batch experiments were performed to study the interaction of MC-LR with phycocyanin over a range of solution pH (2.0–10.8). The pH was adjusted using dilute HCl or NaOH. The samples were equilibrated for 4 h at 300 rpm (Orbit shaker) then centrifuged at 6000 rpm for 1 h using 10 000 molecular weight cut off (MWCO) filter. Typically >95% of the phycocyanin was removed by the MWCO filter based on the absorbance of the filtrate at 618 nm.

Results and Discussion

Effects of Wavelength, MC Structure, and Phycocyanin Concentration on the Phototransformation of MCs

The phototransformations of MCs were studied using UV (350 nm), and visible (450 and >500 nm) light sources. Control experiments show no measurable phototransformation of MC-LR occurs upon irradiation at 350, 450, and >500 nm without phycocyanin. The phototransformation rates using different light sources in the presence of phycocyanin was measured by monitoring the residual concentration of MC-LR as a function of radiation time. The concentration of MC-LR as a function of the cumulated light radiation energy delivered

to the reaction solution is illustrated in Figure 1. At wavelengths of more than 500 nm, MC-LR was not degraded under our experimental conditions. Significant phototransformations of MC-LR occurs at 450 nm, but UV (350 nm) is the most effective for the phototransformation of MCs and has the significant overlap with the UV-A portion of the solar spectrum. For all the subsequent studies the 350 nm light source was employed.

We chose to study three MC variants (MC-LR, MC-RR, and MC-LF) as model compounds for the ~80 MC variants that have been identified. The structures of MC-LR, MC-RR, and MC-LF are shown in Figure 2. The phototransformation of MC-LR, -RR, and -LF in the presence of phycocyanin follows a pseudo first-order decay curve rate (Figure 3). The half-lives of MC-LR, -RR, and -LF under similar experimental conditions are 120 ± 24 min. The structural variations among the different MCs do not appear to have a significant effect on their phototransformation.

Since phycocyanin leads to the photoinduced transformation of MCs, we investigated the effect of phycocyanin concentration on the rate of phototransformation. Based on the ratio of MC to phycocyanin (0.7% weight percentage) measured from the isolated microcystis cells, the concentration of the phycocyanin was varied from 113 to 1020 mg/L. The phototransformation increases significantly as the phycocyanin concentration increases from 113 to 340 mg/L, but further increases in phycocyanin concentration do not enhance the phototransformation rate of MC-LR (Figure 4), most likely the result of the controlled by UV irradiation attenuation parameter (the high optical density of the solution) (16).

Product and Mechanistic Studies

Product studies were conducted employing the optimal reaction conditions established in the previous experiments. An air saturated solution of MC-LR and phycocyanin was irradiated (350 nm). HPLC analysis of the reaction solution indicates a predominant single byproduct is formed from MC-LR (Figure 5). LC-MS analysis indicates the byproduct has the same molecular weight as MC-LR, which suggests the transformation is the result of isomerization or intramolecular reaction. Tsuji et al. (18) and Kaya & Sano (8) reported isomerization of 6 (*E*) to 6(*Z*) MC-LR upon photolysis with 254 nm light. We synthesized an authentic sample of 6(*Z*) Adda MC-LR using 254 nm photolysis, details are provided in the Supporting Information. The chromatographic behavior, UV absorbance and MS of the authentic 6(*Z*) Adda MC-LR (Supporting Information, Figures 3S and 4S) are the same as the byproduct from our photosensitized studies, indicating the major byproduct is 6(*Z*) Adda MC-LR. Direct photolysis using 254 nm light (via direct photoisomerization) and photolysis at 350 nm in the presence of phycocyanin (by a photosensitized process) lead to the formation of 6(*Z*) Adda MC-LR.

One explanation for the observed isomerization under our experimental conditions involves a photosensitized process. As described in the introduction, phycocyanin is excited upon irradiation at 350 nm to form a singlet excited-state which intersystem crosses (ISC) to a triplet. The triplet phycocyanin can undergo energy transfer to the diene moiety of Adda portion of MCs, eq 6. The triplet diene can undergo isomerization from the *E* to the *Z* isomer, eq 7. Cooper et al. proposed an analogous mechanism for the photosensitized transformation of a diene by humic substances (19). While we expect *Z* can isomerize to the *E* form by an analogous process, the *E/Z* ratio will reach a steady state under extended irradiation. The *Z* isomer has much lower toxicity (18) and thus may undergo biodegradation more readily than the *E* isomer.

Photooxidation plays a key role in the photochemical fate of pollutants and naturally occurring substrates in aqueous media. To better understand the role of photooxidation in the transformation of MCs in the environment, a series of experiments was conducted. While our experimental conditions can lead to type I and II photooxidations, our product studies suggest radicals (type I photooxidation) do not play a major role in the phototransformation of MC-LR. The major observed product is the result of double bond isomerization which is also not consistent with type I photooxidation. The type II photooxidation can yield singlet oxygen (20). We proposed photosensitized isomerization of 6(*E*) MC-LR to 6(*Z*) MC-LR earlier, but singlet oxygen can also lead to isomerization of dienes (21).

To explore the role of ground state oxygen and singlet oxygen in the phycocyanin photosensitized transformation of MC-LR, experiments were conducted in the absence of oxygen. The solution was purged with argon to eliminate all oxygen prior to irradiation; under such conditions all oxygen mediated processes are eliminated. While photooxidation is reported to play a significant role in the photosensitized degradation of MCs, the phototransformation of MC-LR is fastest under argon saturation (without oxygen), intermediate under air saturation (~20% oxygen), and slowest under oxygen saturation (100% oxygen), summarized in Table 1. Similar results are observed for MC-RR.

Our results indicate that oxygen is not required for the phototransformations of MCs and, in fact, oxygen can inhibit the process. Under our experimental conditions, the color of phycocyanin faded during irradiation. Photo-bleaching of phycocyanin monitored by 618 nm occurs under Ar, air, and oxygen saturated conditions, transformation rates are summarized in Table 1.

We observed bleaching of phycocyanin increases with increasing concentration of oxygen, indicating a self-sensitized photooxidative process is operative, represented by eqs 8 and 9. Under oxygen saturation, higher yields of singlet oxygen can be produced than under air or argon saturation. Since the concentration of phycocyanin (0.34 mg/mL) is much higher than MCs (2.5 µg/mL), as is the case under environmental conditions, MCs may not effectively compete with phycocyanin for reactions of singlet oxygen, eqs 9 and 10. This suggests that the presence of oxygen during photolysis will lead to the formation of singlet oxygen which degrades the phycocyanin and decreases the extent of photosensitized isomerization of MCs.

The phototransformation of MC-LR decreases with increasing concentration of oxygen, indicating photosensitized isomerization of MC and singlet oxygen formation are competitive processes. Irradiation of an argon-saturated solution of phycocyanin with UV light sharply reduces the photobleaching rate of phycocyanin, while enhancing photosensitized isomerization of MC-LR. The bleaching under argon may be due to the photoinduced phycocyanin dissociation from trimer to monomer, or the direct damage to the aromatic amino acids and bilins of phycocyanin similar to the photodestruction of phycobiliproteins by UV-B radiation (15).

To further probe the role of singlet oxygen, reactions were conducted in the presence of NaN₃, which strongly inhibits singlet oxygen mediated processes (22). A significant increase in the phototransformation rate of MC-LR is observed in the presence of NaN₃ (Figure 6). These results indicate singlet oxygen may not be involved in the phototransformation of MC-

LR. The bleaching rate of phycocyanin in the presence of NaN_3 also decreases significantly, consistent with the proposed photobleaching pathway. Since singlet oxygen has a longer lifetime in deuterated solvents, if singlet oxygen is the principal pathway for photosensitized transformation of MCs, the rate should be faster when experiments are conducted in D_2O compared to H_2O (23). Under our experimental conditions, the degradation rate of MC-LR is slower in the D_2O than in H_2O (Figure 6). These results further support the conclusion that singlet oxygen leads to the degradation of phycocyanin reducing the degradation of MC-LR.

While the levels of phycocyanin and MCs are highest during the breakdown of cyanobacteria bloom, the level of dissolved oxygen is significantly reduced. Under field conditions, dead algae sink to the bottom and stimulate growth of bacteria, which further deplete oxygen in the water bodies and lower oxygen levels in the water body. While the levels of dissolved oxygen and light penetration vary significantly with depth, anaerobic conditions may lead to more effective phototransformation of MCs under specific field conditions.

Effect of pH on the Phototransformation of MC-LR

Since the solution pH can vary depending on the quality of water, the rates of phototransformation of MC-LR as a function of solution pH 3.7–10.3 were studied. The reaction profiles are consistent with pseudo first-order reaction. The pseudo first-order rate constants for the phototransformation of MC-MC-LR increase with decreasing pH, as summarized in Table 2.

The pH effect may be the result of changes in absorptivity of phycocyanin as a function of solution pH. Our results also indicate that energy transfer from sensitizer to MC plays a key role in the phototransformation of MC. With this in mind one might expect that the association of MC and the photosensitizer will have a pronounced effect on the phototransformation and may be influenced by pH. Given that MCs and phycocyanin possess ionizable groups (carboxylic acids with $\text{pK}_a \sim 5$ and amino groups which can exist as protonated forms) the charges associated with these molecules varies with solution pH. Under alkaline pH, both MCs and phycocyanin possess overall negative charges as the carboxylate groups will be anionic and the amino groups will be neutral. Under these conditions it is probable that interaction of MC and phycocyanin will be reduced by electrostatic repulsion. As the solution becomes more acidic the negative charges and electrostatic repulsion are reduced and the interaction of MC with phycocyanin may be enhanced. Molecular weight cutoff filters (MWCO) were used to probe the extent of interaction/aggregation of MCs with phycocyanin as a function of solution pH. Control experiments were conducted and demonstrated that MC-LR alone is not retained by the MWCO over the range of solution pH employed in these experiments. The solution of MC and phycocyanin was adjusted to the desired pH, allowed to equilibrate then subjected to MWCO filtration. The concentration of MC-LR in the filtrate was measured under alkaline, neutral and acidic pHs. Under neutral and alkaline conditions, 100% of the MC-LR passes through the MWCO filter indicating no aggregation or complexation between phycocyanin and MC-LR. As the pH decreases, the amount of MC-LR that passes through the MWCO filter decreases. Phycocyanin is too large to pass through the MWCO filters employed in our studies. The decrease is attributed to strong association between MC-LR and phycocyanin such that MC-LR does not pass through the MWCO filter (Figure 7). We have demonstrated that MC and phycocyanin can form a strong association, such an association may facilitate energy transfer. The association of MC with phycocyanin could occur via a number of mechanisms, including but not limited to the association of MC to the protein portion of the sensitizer, through hydrogen bonding or hydrophobic interactions. We speculate that a close interaction of the sensitizer with MC is favorable for energy transfer, but additional studies are required to assess the nature of the interaction.

In summary, our results demonstrate solar simulated conditions lead to the rapid phototransformation of MCs in the presence of phycocyanin. Product studies using MC-LR indicate the predominant process involves isomerization of the diene (6E to 6Z) in the Adda side chain. The 6Z isomer is known to have dramatically reduced toxicity (18) and thus may be more susceptible to biodegradation. The process appears to involve sensitized isomerization via the photoexcited phycocyanin. Since all MCs variants contain the Adda side chain, we expect this process should be operative for all MCs. Our experiments indicate that oxygen inhibits the photodegradation of MCs and the formation of singlet oxygen leads primary to the bleaching of the photosensitizer. Under algae bloom conditions, the concentration of dissolved oxygen can be quite low, hence, the inhibition of MCs phototransformation by oxygen may be minimized. Our results indicate that the photochemical transformation MCs under simulated environmental conditions occurs predominately by photosensitized isomerization, while photooxidation may play only a minor role. The action leading to the photoexcitation of phycocyanin and subsequent transformation of MCs may account for the low persistence of MCs in the environment.

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FIGURE 1. Phototransformation of MC-LR by UV (350 nm), visible (450 nm) and visible (≥ 500 nm) irradiation. The initial concentration of phycocyanin is 0.34 mg/mL. The reproducibility of the analytical measurements was $\pm 2\%$ on the basis of triplicate runs. The error is based on the standard deviation

FIGURE 2.

Structures of MC-LR, -RR, and -LF. (-LR: X = Leucine, Y = Arginine;-RR: X =Arginine, Y = Arginine;-LF: X = Leucine, Y = Phenylalanine).

FIGURE 3.

Phototransformation of three MC variants (-LR, -RR, and -LF) in aqueous air saturated solutions under 350 nm irradiation. The initial concentration of phycocyanin is 0.34 mg/mL.

FIGURE 4. Phototransformation of MC-LR in air saturated solution by 350 nm irradiation under different initial concentrations of phycocyanin.

FIGURE 5.

HPLC chromatograms and UV spectra of MC-LR and the major product formed upon irradiation of 350 nm light in the presence of phycocyanin (0.34 mg/mL). (a) UV spectrum of MC-LR. (b) UV spectrum of the major product.

FIGURE 6.

Phototransformation of MC-LR in aqueous solution and in the presence of the $^1\text{O}_2$ scavenger NaN_3 (200 mg/L) and in deuterated solvent. Experiments were performed under air saturated conditions. The initial concentration of phycocyanin and MC-LR are 0.34 mg/mL and 2.5 $\mu\text{g}/\text{mL}$ respectively.

FIGURE 7.

MC-LR complexation with phycocyanin as a function of solution pH. The MC-LR recovered percentage is the fraction of MC-LR that passed through the MWCO filter. The initial concentration of phycocyanin and MC-LR are 0.34 mg/mL and 2.5 μ g/mL, respectively. Analyses were performed in triplicate, and the error bars indicate the standard deviation of the mean.

TABLE 1

Pseudo First-Order Rate Constants for Transformation of MC-LR and Phycocyanin under Oxygen, Air and Argon Saturated Conditions

saturating gas	$k_{\text{MC-LR}} (10^{-3} \text{ min}^{-1})^b$	R^2	$k_{\text{phycocyanin}} (10^{-3} \text{ min}^{-1})^b$	R^2
oxygen ^a	<0.1		33.0 ± 0.5	0.999
air ^a	7.0 ± 0.5	0.999	20.2 ± 1.3	0.992
argon ^a	22.4 ± 2.0	0.994	9.0 ± 0.9	0.969

^aThe solution was purged with appropriate gas for 5 min with a flow rate ~30 mLs/min before UV irradiation. The reaction vessel was sealed during UV irradiation.

^b Observed first-order exponential decay rate (min^{-1}). The reproducibility of the individual data points is within 5% based on triplicate runs. The error is based on the standard deviation.

TABLE 2

Pseudo First-order Rate Constants Photosensitized Transformation of MC-LR at Different pH

pH of the solution	$k_{\text{MC-LR}} (10^{-3} \text{ min}^{-1})^a$	R^2
3.7	10.5 ± 0.5	0.999
4.7	8.6 ± 0.6	0.999
7.0	7.0 ± 0.5	0.999
8.5	6.6 ± 1.0	0.996
10.3	3.3 ± 0.8	0.997

^a Observed pseudo first-order exponential decay rate (min^{-1}). The error is based on the standard deviation.