Ammonia has long been known to be toxic for many photosynthetic organisms; however, the target for its toxicity remains elusive. Here, we show that in the cyanobacterium *Synechocystis* sp. strain PCC 6803, ammonia triggers a rapid photodamage of photosystem II (PSII). Whereas wild-type cells can cope with this damage by turning on the FtsH2-dependent PSII repair cycle, the FtsH2-deficient mutant is highly sensitive and loses PSII activity at millimolar concentration of ammonia. Ammonia-triggered PSII destruction is light dependent and occurs already at low photon fluence rates. Experiments with monochromatic light showed that ammonia-promoted PSII photoinhibition is executed by wavebands known to directly destroy the manganese cluster in the PSII oxygen-evolving complex, suggesting that the oxygen-evolving complex may be a direct target for ammonia toxicity.

Ammonium (in this communication, the term ammonium denotes both NH$_3$ and NH$_4^+$) is a molecule of fundamental importance to autotrophic organisms because the assimilation of nitrogen into organic material occurs through ammonium-dependent reactions. Ammonium is either directly taken up from exogenous sources or the organisms employ reaction pathways that generate ammonium from other nitrogenous compounds. On the other hand, ammonium is known to be toxic for many organisms, in particular to plants and oxygenic photosynthetic microorganisms. A variety of herbicides (such as glufosinate) act as inhibitors of the key enzyme of ammonium assimilation, Gln synthetase, thereby leading to the accumulation of toxic concentrations of ammonium. It is widely believed that ammonium toxicity is caused by the uncoupling of photophosphorylation; however, this assumption could be proven to be wrong for whole organisms (Zhu et al., 2000; Britto and Kronzucker, 2002). More recent investigations in higher plants suggested that futile transmembrane NH$_4^+$ cycling might be involved in ammonium sensitivity (Britto et al., 2001). Furthermore, ammonium toxicity is more pronounced under high light conditions, an observation that has not been explained so far (Zhu et al., 2000; Britto and Kronzucker, 2002). In contrast to plants, heterotrophic bacteria seem to be highly resistant to ammonium because they were shown to tolerate ammonium concentrations until they suffer osmotic stress (Müller et al., 2006). Cyanobacteria, a domain of bacteria that performs oxygenic photosynthesis, display metabolic properties similar to plants. Although, to our knowledge, ammonium sensitivity has so far not been systematically investigated in cyanobacteria, higher concentrations of ammonium are toxic to many strains.

The cyanobacterium *Synechocystis* sp. strain PCC 6803 (hereafter called *Synechocystis*) is widely used to study fundamental processes of photosynthetic metabolism. As for many cyanobacteria, ammonium is the preferred nitrogen source for *Synechocystis*. The uptake of ammonium is affected by the pH-dependent equilibrium between NH$_3$ and NH$_4^+$. Whereas ammonium (NH$_3$), which prevails under alkaline conditions, can diffuse through membranes, the protonated ion (NH$_4^+$) cannot (Kleiner, 1981). Ammonium at low concentrations is taken up by high affinity transporters of theAmt family, whose activity is regulated by the nitrogen state of the cells (von Wiren and Merrick, 2004). Energy metabolism of *Synechocystis* depends on oxygenic photosynthetic electron transport, involving two types of reaction centers: PSI and PSII. The oxidation of water takes place in the oxygen-evolving complex (OEC) of PSII (Debus, 1992; Diner, 2001; Ferreira et al., 2004). The PSII structure of the cyanobacterium *Thermosynechococcus elongatus* revealed that...
the OEC consists of a cubane-like Mn$_3$CaO$_4$ cluster and a fourth manganese (Mn) atom linked to the complex by one of its oxygen atoms. The Ca$^{2+}$ and Cl$^-$ ions, which are associated with the OEC, are participating in deprotonation of the substrate water molecules and in stabilization of the intermediates (Ferreira et al., 2004; Iwata and Barber, 2004; Barber, 2006). The metal ions of the OEC are tightly liganded to residues of the D1 protein of PSII; the C-terminal domain of D1 is involved in stabilization of the OEC and important for the first steps of the assembly of the Mn$_3$Ca cluster (Diner et al., 1991; Cohen et al., 2007).

The D1 protein of PSII itself is sensitive to light-induced damage, leading to loss of PSII function. The mechanistic basis of PSII photoinhibition has been an issue of controversial discussion. Recent evidence strongly suggests that photooxidation of the OEC is the initial reaction, which together with subsequent reactions leads to photodamage. The absorption of short wavelength blue and UV light by the Mn cluster causes Mn release from OEC and thereby its inactivation (Hakala et al., 2005; Ohnishi et al., 2005). When the reaction center chlorophyll $a$ (chl$a$; donor P680) becomes photooxidized, the highly reactive P680$^+$ cannot be reduced from water oxidation and damages the D1 protein (Hakala et al., 2005, 2006; Ohnishi et al., 2005; Murata et al., 2007). Reactivation of PSII involves an efficient PSII repair cycle; the damaged D1 protein is triggered for removal and degradation by a membrane-bound ATP- and Zn$^{2+}$-dependent FtsH protease, which is a member of the AAA protease family. Following removal of inactive D1, newly synthesized D1 protein is assembled in PSII, ensuring proper photosynthetic electron transport (Aro et al., 1993; Komenda and Barber, 1995; Nixon et al., 2005). Interestingly, the genome of Synechocystis encodes four putative FtsH homologs (Kaneko et al., 1996). Out of these, the $ftsH2$ (slr0228) gene was shown to encode the protease responsible for D1 degradation (Silva et al., 2003; Komenda et al., 2006). Apart from its role in D1 turnover, FtsH2 was also shown to be involved in degradation of various soluble enzymes in Synechocystis (Stirnberg et al., 2007). This study was initiated to investigate the role of FtsH2 in protein turnover under changing nitrogen regimes. In the course of this investigation, FtsH2 turned out to be essential for acclimation of Synechocystis toward ammonium. Subsequent investigations revealed a novel mechanism of ammonium toxicity in photosynthetic organisms, namely, photosensitizing PSII.

### RESULTS AND DISCUSSION

The $ftsH2$ (slr0228) Gene Is Required for Ammonium Tolerance of Synechocystis

According to the participation of FtsH2 in PSII repair, the $ftsH2^-$ mutant is sensitive to higher photon flux densities (above 40 $\mu$mol photons m$^{-2}$ s$^{-1}$) in standard BG-11 medium (nitrate supplemented) and is affected in pigmentation as well as in photosynthetic electron transport. Therefore, under standard growth conditions (liquid cultures in carbonate-buffered BG-11 medium, containing 17.6 mM NaNO$_3$), the $ftsH2^-$ mutant cells already display a lower photosynthetic activity than wild-type cells (Mann et al., 2000; Silva et al., 2003). Initial experiments indicated that the $ftsH2^-$ mutant is highly sensitive toward ammonium chloride. When exposed to 5 mM NH$_4$Cl at a photon flux density of 20 $\mu$mol photons m$^{-2}$ s$^{-1}$, the cells rapidly lost photosynthetic activity (see below) and viability. After 24 h in the presence of NH$_4$Cl, the culture of the $ftsH2^-$ mutant was completely bleached (data not shown) and the cells were unable to reinitiate growth on standard BG-11 plates with nitrate as nitrogen source, whereas cells cultured in parallel in BG-11 in the absence of NH$_4$Cl were unaffected (Fig. 1A). Further, in contrast to the wild type, the $ftsH2^-$ mutant was unable to grow on BG-11 NH$_4$Cl-supplemented plates (Fig. 1B). To
study the effect of NH₄Cl on photosynthetic activity, PSII activity was measured by two independent methods (Fig. 2): (1) by measuring light-saturated rates of oxygen evolution using a Clark electrode; and (2) by using the modulated chlorophyll fluorescence method in a PAM chlorophyll fluorometer, which allows determination of the electron transport rate (ETR) through PSII (for details, see "Materials and Methods"). Following the addition of 5 mM NH₄Cl to the BG-11-grown cultures, a rapid decline in PSII activity reaching zero level within several hours was measured in the \textit{ftsH}²⁻ mutant by both methods, whereas in nontreated control cultures, PSII activity remained unaffected. The wild type also displayed an initial drop in PSII activity following ammonium chloride addition; however, its PSII activity gradually recovered after 2 to 4 h. Because both PSII measurements reported a similar response of activity, in all subsequent experiments, PSII activity was recorded only by the more convenient PAM measurements.

To distinguish between ammonia (NH₃) or protonated ammonium ions (NH₄⁺) as causative agents of PSII activity loss in the \textit{ftsH}²⁻ mutant, the experiment was performed in the presence of different buffer and pH conditions (see Table I), making use of the pH-dependent equilibrium between NH₃ and NH₄⁺ (pKₐ of 9.2). When the medium was buffered with 20 mM HEPES to a pH of 8.2, the mutant cells maintained 63% of their initial PSII activity after 5-h incubation. More alkaline conditions led to a stronger decrease of PSII activity, and at pH 8.8, PSII activity was completely lost after 5-h incubation. Controls carried out under the same illumination conditions showed that the PSII activity in the \textit{ftsH}²⁻ mutant was unaffected in the presence of nitrate or nitrite under any pH conditions tested (Table I shows the most alkaline condition tested). Apparently, the loss of PSII activity that was observed in the \textit{ftsH}²⁻ mutant correlates with the free ammonia concentration (Table I). When ammonium is present in the protonated form, its uptake is accom-

![Figure 2](image-url). Effect of ammonium addition on PSII activity. Cultures of wild type (white symbols) and \textit{ftsH}²⁻ (black symbols) grown in standard BG-11 medium to mid-exponential phase of growth were treated with 5 mM NH₄Cl (squares) at time zero. As control, cells were grown in standard BG-11 medium without addition (circles). Cells were illuminated with 20 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) of white light, and at the indicated time points, samples were removed for determination of PSII activity by tow different methods. A, Measurement of light-saturated rates of oxygen evolution. B, ETR through PSII by PAM chlorophyll fluorometry as described in "Materials and Methods." The values are the averages of three independent experiments and the error bars indicate sd.

<table>
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<tr>
<th>Media and Buffer Conditions</th>
<th>pH</th>
<th>Free Ammonia Concentration in Medium (( \text{mM} ))</th>
<th>Free Ammonia Concentration in Medium (%)</th>
<th>PSII Activity after 5-h Incubation (%)</th>
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<tr>
<td>BG-11 + 5 mM NH₄Cl/carbonate</td>
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<td>1.45</td>
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<tr>
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<td>0.45</td>
<td>63</td>
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<tr>
<td>BG-11 (17.6 mM NaNO₃)/carbonate</td>
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<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>BG-11 + 10 mM NO₂/carbonate</td>
<td>9.1</td>
<td>0</td>
<td>74</td>
<td></td>
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plished by ammonium transporters of the Amt family, whose activity is regulated by the nitrogen state of the cells. Ammonium-rich conditions cause inhibition of Amt activity (Montesinos et al., 1998), which avoids the accumulation of excess intracellular ammonium. However, when ammonia freely diffuses through membranes, the cells are not able to control its uptake. Under those conditions, ammonia causes damage to PSII in the ftsH2 wild-type cells. To determine the concentration of ammonia that can be tolerated by wild-type and mutant cells, experiments carried out as outlined above were performed in the presence of different concentrations of NH₄Cl at a constant pH of 8.8, where a substantial amount of ammonium is present as nonprotonated ammonia. Figure 3A shows that the PSII activity in Synechocystis wild-type cells was not affected by up to 10 mM NH₄Cl. Higher concentrations led to a gradual decrease of PSII activity, but even at a concentration of 60 mM NH₄Cl, the cells maintained an appreciable PSII activity of more than 20% compared to untreated cells. By contrast, the mutant cells were highly susceptible, because already in the presence of 1 mM NH₄Cl, they lost 40% of their photosynthetic activity and at 5 mM NH₄Cl, no more variable chlorophyll fluorescence could be measured. Therefore, the optimal conditions to study the effect of ammonia on the FtsH2 mutant turned out to be 5 mM NH₄Cl at a pH of 8.8, which was used in the subsequent studies.

Ammonia-Mediated Damage of PSII in the FtsH2-Deficient Mutant Is Light Dependent

To unravel whether the observed loss of PSII function in the presence of NH₄Cl in the ftsH2 wild-type cells is a light-dependent process, the time course of PSII inhibition was investigated at three different photon fluence rates and in the dark (Fig. 3B). During exposure of the ftsH2 mutant to 5 mM NH₄Cl in the presence of 10 µmol photons m⁻² s⁻¹ of white light, the PSII activity dropped to 40% after 60 min and was almost completely lost after 220 min. In the presence of 20 µmol photons m⁻² s⁻¹, the cells already lost 70% of their initial ETR during the first 40 min, and after 180 min...

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**Figure 3.** Ammonium tolerance of Synechocystis wild-type and ftsH2 mutants. A, NH₄Cl concentration-dependent impairment of PSII activity. Cultures were grown in standard BG-11 medium to an OD₇₅₀ of 0.5 under continuous irradiation with white light at a fluence rate of 10 µmol photons m⁻² s⁻¹. Ammonium chloride was then added to the cultures in different concentrations, and the cultures were further incubated for 5 h in presence of 20 µmol photons m⁻² s⁻¹. Samples were taken for measuring PSII activity (relative ETR, see "Materials and Methods"). B, Fluence rate-dependent damage of PSII in the presence of 5 mM ammonium chloride. Cultures of wild-type and ftsH2 mutants were grown in standard BG-11 medium (OD₇₅₀ of 0.5) under illumination with white light at a fluence rate of 10 µmol photons m⁻² s⁻¹. Ammonium chloride (5 mM) was added to the culture at time point zero and incubated with different light intensities (10 [squares], 20 [triangles], or 40 [diamonds] µmol photons m⁻² s⁻¹) or in darkness (circles). Samples were taken at the indicated time points for measuring relative ETR. The data in parts A and B are the means of three independent experiments, with error bars showing the SD.
the PSII activity was completely lost. In the presence of 40 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \), loss of PSII activity was even faster. However, when the mutant cells were exposed to NH\(_4\)Cl in the absence of light, the cells were almost not affected. In contrast to the mutant, the wild-type cells were only transiently impaired in PSII activity following the addition of NH\(_4\)Cl. After an initial drop in the first 60 min by 30% to 50% compared to the untreated control, the cells overcame the stress and recovered photosynthetic activity nearly completely after 2 to 3 h. The initial response suggests that ammonia damages PSII in the wild type as well; however, in the presence of functional FtsH2, PSII activity can be efficiently reactivated. Furthermore, the damaging effect of ammonia is clearly enhanced by increasing light intensity, suggesting that photodamage and FtsH2-dependent PSII repair is responsible for the response of PSII activity toward ammonia.

Ammonia Tolerance in Wild-Type Cells Depends on a Functional PSII Repair Cycle

The degradation of the D1 protein by the FtsH2 protease is a key process in the PSII repair cycle, which mediates recovery from photoinhibition (Aro et al., 1993; Nixon et al., 2005). To investigate whether the ammonia sensitivity in the ftsH2-deficient mutant is indeed due to impaired PSII repair, protein synthesis in wild-type and mutant cells, treated with or without NH\(_4\)Cl, was inhibited by chloramphenicol. In the absence of de novo protein synthesis, no PSII repair cycle occurs. When protein synthesis was inhibited in NH\(_4\)Cl-treated wild-type cells, they rapidly lost PSII activity and became almost as sensitive toward light as the ftsH2~ mutant (Fig. 4A). In the NH\(_4\)Cl-exposed ftsH2~ mutant, inhibition of protein synthesis did not further accelerate the loss of PSII activity compared to cells in which protein synthesis was not inhibited (compare Figs. 3B and 4A). In contrast, when at the same light intensity NaNO\(_3\) was present instead of NH\(_4\)Cl, only a moderate inhibition of PSII was observed in the wild type and in ftsH2~ mutant cells (Fig. 4B).

To directly reveal the effect of ammonia on the degradation of D1, western-blot analysis with antibodies against PsbA (D1 protein) were performed in chloramphenicol-treated wild-type and mutant cells. In wild-type cells, the D1 protein was completely degraded in the presence of NH\(_4\)Cl, whereas it was much more stable in the presence of NaNO\(_3\) (Fig. 5). This feature indicates that ammonia indeed accelerates D1 destruction, as visualized by the complete D1 removal in wild-type cells. As expected, the FtsH2-deficient mutant was not able to degrade the D1 protein, and therefore the protein levels remained high in both nitrogen regimes (Fig. 5), confirming that the rapid disappearance of D1 in the wild type in the presence of NH\(_4\)Cl was due to FtsH2-mediated activity. Altogether, this analysis shows that ammonia indeed accelerates PSII photodamage even at low light intensity (20 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \)), an effect that...
becomes evident only when the PSII repair cycle is inhibited, either by inhibiting de novo protein synthesis or by deleting the FtsH2 protease.

Additional evidence that ammonia enhances photoinhibition by stimulating photodamage and not by impairing the repair of photodamage was obtained by a set of experiments performed with *Synechocystis* wild-type cells. Cells were exposed to photoinhibitory light conditions (1,500 photons m\(^{-2}\) s\(^{-1}\)) in the absence or in the presence of 5 mM NH\(_4\)Cl. Samples were taken after different time points to determine the quantum yield of PSII (Y) by PAM chlorophyll fluorescence, which is a reliable indicator of photoinhibition. As shown in Figure 6A, photoinhibition (as revealed by the decrease in PSII quantum yield) is clearly accelerated in the presence of NH\(_4\)Cl. A similar result was obtained by measuring oxygen evolution under photoinhibitory high light conditions; under light conditions in which oxygen evolution decayed within 15 min, the addition of 5 mM NH\(_4\)Cl lead to the cessation of photosynthetic oxygen evolution in less than 1 min (not shown). To assess whether NH\(_4\)Cl could affect the repair of photodamaged PSII, cells were first exposed to photoinhibitory conditions for 12 min in the presence of 5 mM NH\(_4\)Cl, where the PSII quantum yield had dropped to zero. Then, the cells were transferred to fresh medium and allowed to recover from photoinhibition at low light intensities (20 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) in the presence or absence of 5 mM NH\(_4\)Cl. As shown in Figure 6B, recovery from photoinhibition was not affected by the presence of NH\(_4\)Cl, whereas the addition of chloramphenicol completely inhibited the recovery from photodamage, indicating that recovery required the synthesis of novel protein. In conclusion, this experiment demonstrates that NH\(_4\)Cl has no detrimental effect on the recovery of PSII from photodamage.

Because the FtsH2 protease seems to be crucial for ammonia tolerance of *Synechocystis* wild-type cells, the expression of the ftsH2 gene in response to NH\(_4\)Cl treatment was investigated. The abundance of ftsH2 mRNA was analyzed by northern-blot DNA-RNA and slot-blot RNA-RNA hybridization, revealing a significantly increased level of the ftsH2 (srl0228) transcript in ammonium-treated cells in contrast to cells grown on nitrate (Fig. 7). The ftsH2 transcript abundance was increased approximately 2.5-fold (densitometrically estimated from RNA-RNA hybridization signals) after the addition of NH\(_4\)Cl and remained elevated after 1 h.

**The Primary Target of Ammonia-Induced PSII Photoinhibition May Be the OEC**

To clarify the mechanism of coaction of light and ammonia in PSII destruction, a rough action spectrum of ammonia-induced photoinhibition of PSII was taken. Mutant cells, and wild-type cells as control, were illuminated with monochromatic light of different wavelengths from 360 to 700 nm in the presence of NH\(_4\)Cl, and the photosynthetic activity of PSII was monitored.

![Figure 6](image)

**Figure 6.** Ammonium-dependent PSII photodamage and repair in *Synechocystis* wild-type cells. A. *Synechocystis* wild-type cells were diluted in standard BG-11 nitrate medium to an OD\(_{750}\) of 0.3. Three-milliliter aliquots were removed into glass test tubes, supplemented (white triangles) or not supplemented (black circles) with 5 mM NH\(_4\)Cl, and placed in the light beam of a halogen lamp at a fluence rate of 1,500 photons m\(^{-2}\) s\(^{-1}\). At indicated time points, 100-\(\mu\)L aliquots were withdrawn for assessment of PSII quantum yield. For this purpose, the 100-\(\mu\)L samples were diluted into 1.9 mL of BG-11 nitrate medium and transferred into the measuring cuvette of a WATER-PAM chlorophyll fluorometer. After 2 min in the dark measuring cell, the measuring beam was switched on for determination of \(F_0\). Saturating light pulses were applied to determine \(F_{\infty}\) and to quantify quantum yield of PSII (Y) from the \(F_0/F_{\infty}\) ratio as described by Schreiber et al. (1995). B. The 8-mL samples of *Synechocystis* wild-type cells were photodamaged for 12 min in the presence of 5 mM NH\(_4\)Cl as described in A. The damaged cells were gently transferred to ammonium-free, nitrate-supplemented BG-11 medium by 5 min centrifugation at 1,400 \(g\), twice washing the pellet with 8 mL ammonium-free BG-11 nitrate medium. After the last centrifugation, the cells were resuspended in 8 mL BG-11 nitrate medium. To one sample, chloramphenicol was added (30 \(\mu\)g/mL final concentration) to inhibit novel protein synthesis (black squares). The samples were incubated in Erlenmeyer flasks with a photon fluence rate of 20 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\). After different time points as indicated, 100-\(\mu\)L aliquots were withdrawn for determination of PSII quantum yield as described above. After 40 min, at the onset of recovery in the absence of chloramphenicol, 5 mM NH\(_4\)Cl was added to one sample (indicated by the arrow), and the samples were further incubated to record the recovery from photoinhibition in the absence (black circles) or presence (white triangles) of NH\(_4\)Cl. Triplicate experiments yield almost identical results (SD < 5%). All experimental steps were performed at 25°C.
The strongest effects, however, were observed with short wavelength blue and near UV (UV-A) irradiation. Illumination with light at 421 nm, 393 nm, and 360 nm caused significantly increasing damage of PSII, with PSII inhibition rates of 30%, 60%, and 85%, respectively (Fig. 8). As expected from their ammonium-tolerance, PSII activities in the wild-type cells were not severely affected by the addition of NH₄Cl nor was the ftSH₂⁻ mutant affected, when illuminated in the presence of nitrate (Fig. 8).

Photoinhibition was previously suggested to be mediated by two different photosensitizers: in addition to a contribution of chl, a photosensitizer that absorbs blue/UV light is responsible for the high sensitivity of PSII toward UV-A and UV-B light (Greenberg et al., 1989; Ohnishi et al., 2005). The blue light/UV photosensitizer was originally suggested to be the plastosemiquinone anion radical (Greenberg et al., 1989). However, the blue light/UV sensitivity of PSII in isolated thylakoids disappeared following the removal of the OEC together with its Mn ions, despite that plastosemiquinone was still present (Hakala et al., 2005). More recently, it was suggested that blue light/UV absorption by the OEC causes release of Mn ions and subsequent photoinhibition by destruction of D1, in agreement with the similarity of the absorption spectrum of a Mn(III/IV) model compound and the action spectrum of photoinhibition (Hakala et al., 2005; Ohnishi et al., 2005). Following the destruction of the OEC, excitation of chl would lead in a second step to the generation of long-lived P₆₈₀⁺ radicals that ultimately damages D1. Apparently, the coaction of ammonia with light is confined to the blue/UV light-absorbing photosensitizer, whereas the lack of response to red light (680 and 700 nm) rules out a direct coaction of ammonia with the reaction center chlα molecules. If the blue/UV light photosensitizer is indeed the Mn cluster in the OEC as suggested by Hakala et al. (2005) or Ohnishi et al. (2005), then the direct target of ammonia is the OEC. This suggestion is in accordance of the action spectrum of ammonia-triggered photodestruction with the action spectrum of OEC photodamage (Hakala et al., 2005; Ohnishi et al., 2005). Furthermore, biochemical studies on isolated PSII particles showed previously that ammonia is a competitive inhibitor of the water-splitting reaction. Ammonia is a known inhibitor of water oxidation because it binds to the OEC as structural analog of the substrate water (Evans et al., 2005; Fang et al., 2005), and it was proposed that ammonia is incorporated into the Mn complex as a bridging ligand after 240 min of irradiation. As a further control, the ftSH₂⁻ mutant illuminated in the presence of nitrate was used. As shown in Figure 6, irradiation with light from 440 to 700 nm led to only a slight decrease of PSII activity (4%–25% inhibition) in the NH₄Cl-treated ftSH₂⁻ mutant. Red light (680 nm, 700 nm, absorbed by chl) showed no effect on PSII destruction, whereas orange light of 621 nm, absorbed by phycobiliproteins, caused a slight activity loss of 25% in the ftSH₂⁻ mutant. The strongest effects, however, were observed with short wavelength blue and near UV (UV-A) irradiation. Illumination with light at 421 nm, 393 nm, and 360 nm caused significantly increasing damage of PSII, with PSII inhibition rates of 30%, 60%, and 85%, respectively (Fig. 8). As expected from their ammonium-tolerance, PSII activities in the wild-type cells were not severely affected by the addition of NH₄Cl nor was the ftSH₂⁻ mutant affected, when illuminated in the presence of nitrate (Fig. 8).

Figure 7. Ammonium-dependent accumulation of ftSH2 transcript. A, Northern-blot analysis of ftSH2 transcript in NH₄Cl-treated Synechocystis wild-type cells. Samples were taken from cells grown in BG-11 medium containing 17.6 mM NaNO₃ as control (C) or following the addition of 5 mM NH₄Cl for 1 h (NH₄). RNA was isolated and northern blot was performed as described in “Materials and Methods.” The hybridization signals of ftSH2 and rnpB, which serves as a loading control, were visualized by phosphorimaging. B, RNA-RNA slot-blot analysis of ftSH2 transcript in NH₄Cl-treated Synechoystis wild-type cells. Cells were treated as above, except that one sample was already taken after 30 min. RNA was isolated and slot blot was performed as described in “Materials and Methods.” The hybridization signals of ftSH2 and rnpB, which serves as a loading control, were visualized by a chemiluminescence imager workstation.

Figure 8. Wavelength-dependent inhibition of PSII activity in the presence or absence of ammonium. Exponentially grown cultures of ftSH₂⁻ were treated with 5 mM NH₄Cl (black circles). For controls, exponentially grown cultures of wild-type cells were treated with 5 mM NH₄Cl (black squares) and ftSH₂⁻ culture was grown in standard BG-11 medium containing 17.6 mM NaNO₃ (white circles). Cells were illuminated with the indicated wavebands at 5 μmol m⁻² s⁻¹ for 240 min before relative ETR was determined. The values are the means of three independent experiments with error bars showing the so. The 100% values correspond to the values determined at 462 nm.
Ammonia Toxicity in Cyanobacteria

CONCLUSION

This investigation revealed the importance of the PSII repair cycle in allowing phototrophic growth in the presence of millimolar concentrations of ammonia under alkaline conditions in the cyanobacterium *Synechocystis*. In general, cyanobacteria are alkalophilic and grow readily in media up to pH values of 10. Alkaline conditions with elevated ammonia concentrations might arise in natural environments, e.g. from urea decomposition in the presence of urease-positive bacteria; in the natural habitat of *Synechocystis*, such conditions might occur temporarily. Because free ammonia diffuses through biological membranes, its uptake cannot be controlled by the global nitrogen regulatory system, and, consequently, ammonia can reach intracellular concentrations, which are potentially harmful to cells. In the absence of the FtsH2 protease, the cells are highly susceptible toward these ammonia concentrations in a light-dependent manner, whereas the FtsH2-bearing wild-type cells are able to tolerate appreciable concentrations of ammonia in the light. Clear evidence is presented that shows the PSII activity in *Synechocystis* is affected by ammonia through accelerated photodamage and not through impaired PSII repair. Whereas the wild type is able to compensate the increased damage by elevating the expression of *ftsH2* and by carrying out an efficient repair cycle, the FtsH2-deficient mutant is unable to do so and, therefore, dies upon prolonged incubation with ammonium in the light. In agreement, prevention of the repair cycle by inhibition of de novo protein synthesis causes similar ammonium sensitivity in the wild type as in the *ftsH2* mutant.

Examination of the upstream region of the *ftsH2* gene failed to reveal a consensus-binding motif for NtcA, the global nitrogen control transcription factor in cyanobacteria. All genes regulated by NtcA (whereby NtcA can act as inducer or repressor depending on the promoter architecture) display a characteristic DNA sequence motif (Herrero et al., 2004). The absence of such a motif suggests that the increased expression of *ftsH2* upon ammonium addition is not a response mediated by NtcA but is probably a consequence of increased photoinhibition caused by ammonium. The increased expression of *ftsH2* may enhance the capacity of the repair cycle and contribute to ammonium tolerance. The mechanism of ammonia-induced *ftsH2* gene expression is intriguing and deserves further investigation.

Although this study reveals a novel target for ammonium toxicity, it remains to be elucidated whether ammonium-triggered PSII photodestruction can be generalized to all oxygenic photosynthetic organisms. Two so-far unconnected observations argue in favor of a general significance. First, the toxicity of ammonia in higher plants is frequently more pronounced at high light intensity (Britto and Kronzucker, 2002), implying that a photoinhibitory process is involved. Second, ammonia was shown in vitro to be a potent inhibitor of the water-splitting reaction in PSII preparations from plants by binding to the Mn cluster (Britto et al., 2004; Evans et al., 2005). These findings are in perfect agreement with the photosensitizing effect of ammonia described here for *Synechocystis* and suggest that ammonium toxicity could operate in many oxygenic photosynthetic organisms by photosensitizing PSII. Further biochemical studies on isolated PSII particles are required to clearly elucidate the molecular details of the coaction of ammonia with the blue light/UV absorbing photosensitizer that mediates PSII photodamage. Eventually, such investigations could provide further insights into the various steps involved in PSII photodamage and may help to clarify the role of the OEC in this important biological process.

MATERIALS AND METHODS

Growth of Cyanobacteria

The transformable wild-type strain of *Synechocystis* sp. strain PCC 6803 (hereafter referred to as *Synechocystis*) and the derived *ftsH2* mutant (stlr0228::Km<sup>5</sup>; Stirnberg et al., 2007) were used in this study. All cells were grown photoautotrophically in liquid BG-11 medium containing 17.6 mM Na<sub>2</sub>N<sub>3</sub>O<sub>4</sub> (Rippka, 1988) supplemented with 5 mM NaHCO<sub>3</sub> at 28°C and under continuous light of 10 μmol photons m<sup>−2</sup> s<sup>−1</sup> from white fluorescent tubes (LUMILUX de Luxe Daylight; Osram). They were constantly shaken at 140 rpm. The mutant was maintained with kanamycin (80 μg mL<sup>−1</sup>).

For conditioning with ammonium, the mid-exponential-phase cultures were treated with 5 to 60 mM NH<sub>4</sub>Cl and illuminated with 10 to 40 μmol photons m<sup>−2</sup> s<sup>−1</sup> from white fluorescent tubes (LUMILUX de Luxe Daylight; Osram). They were constantly shaken at 140 rpm. The mutant was maintained with kanamycin (80 μg mL<sup>−1</sup>).

Assay of Survival in the Presence of Ammonia

To assay the ability of the cells to survive in the presence of ammonia for various time periods, 10 μL of the ammonium-treated cell culture (OD<sub>750</sub> of 0.4) were dropped on a BG-11 agar plate, containing 17.6 mM Na<sub>2</sub>N<sub>3</sub>O<sub>4</sub>, 0.3% sodium thiosulfate, and supplemented with 5 mM NaHCO<sub>3</sub> (for the mutant the agar plates were supplemented with kanamycin [50 μg mL<sup>−1</sup>]). The plates were incubated at 28°C and under continuous light of 20 μmol photons m<sup>−2</sup> s<sup>−1</sup> from white fluorescent tubes (LUMILUX de Luxe Daylight; Osram). After 7 d of incubation, the plates were photographed. To assay the ability to grow in the presence of ammonia, BG-11 nitrate agar plates were prepared that were supplemented with 5 mM sodium thiosulfate and ammonia chloride. Ten microliter of mid-exponential-phase cell culture at the OD<sub>750</sub> of 0.4 were dropped on the agar plate and incubated as described above.

Measurements of Chlorophyll Concentration

The cells (1 mL) were sedimented by centrifugation (20,000g, 3 min, room temperature) and resuspended in 1 mL 99% methanol. The suspension was
incubated for 30 min on ice and in the darkness. Afterward the cell debris was sedimented (20,000 g, 1 min, room temperature) and the clear supernatant was used for absorption measurements (665 nm). The concentration of chlorophyll was calculated from absorbance of the supernatant at 665 nm according to Mackinney (1941).

Oxygen Evolution Measurement

Photosynthetic oxygen evolution was measured in vivo using a Clark-type oxygen electrode (Hansatech DW1). Light was provided from a high-intensity white-light source (Hansateh L2). Oxygen evolution was measured at room temperature of 2 mL of mid-exponential-phase cell culture at the OD750 of 0.4 using the Hill reaction. The culture was illuminated with 3,000 μmol photons m⁻² s⁻¹ in the presence of an artificial electron acceptor system of 1 mM 2,5-dimethyl-p-benzoquinone and 1 mM potassium ferricyanide.

Measurement of Photosynthetic Activity by PAM Chlorophyll Fluorescence

PSII activity was analyzed in vivo in a WATER-PAM chlorophyll fluorometer (Walz GmbH). The chlorophyll fluorescence (Fₐ) of dark-adapted cells was determined and the effective quantum yield (Y) of PSII photochemistry with actinic light of 655 nm (55 μmol photons m⁻² s⁻¹) was determined with the saturation pulse method (Genty et al., 1989; Schreiber et al., 1995). The relative ETR was calculated from the quantum yield (Y) as described previously (Sauer et al., 2001). Cultures were diluted 1:10 in BG-11 medium before the measurements in a final volume of 2 mL.

Monochromatic Light Treatments

Five milliliters of mid-exponential-phase cell cultures were illuminated in petri dishes with monochromatic light through interference filters (Schott). The half bandwidth of all filters was 14 nm or less. The fluence rate during monochromatic light treatments was set to 5 μmol m⁻² s⁻¹. Energy fluence rates were determined using a P-2000 photometer combined with a calibrated PD-9006-2 detector (Gigahertz) and photon fluence rates calculated from these measurements.

Gel Electrophoresis and Immunoblot Analysis

Cell extracts were prepared by vortexing the cells with glass beads (110-μm diameter) followed by centrifugation (3,500 g, 5 min, 4°C). Protein composition was assessed by electrophoresis in a denaturing 12.5% SDS-PAGE containing 6 μL urea. Then 7 μg of total protein per lane was loaded. After electrophoresis, proteins were blotted onto nitrocellulose membranes. The membrane was incubated with chicken anti-PsbA antibody (Agrisera) followed by incubation with rabbit anti-chicken IgG antibody (Agrisera) and the bands visualized by colorimetric reaction using Lumilight for western blotting (Roche).

ftsH2 Transcript Quantification Experiments

For analysis of ftsH2 mRNA abundances under different nitrogen sources, Synecochysis wild-type cells grown in BG-11 medium with 17.6 mM NaNO₃ were treated with or without 5 mM NH₄Cl for 60 min. Thereafter, 50 μL of the treatment and RNA extracted by subsequent use of the NucleoSpin RNA II kit (Macherey und Nagel) according to the manufacturer’s instructions. Two micrograms of total RNA was denatured by treatment with formamide, loaded onto a nitrocellulose membrane, and covalently bound by UV cross-linking. Probes were obtained by PCR amplification of DNA probes internal to the plasmid with the cloned ftsH2_1 and ftsH2_2 gene (ftsh2 and m9p9 with chromosomal DNA of Synecochysis as template and ftsH2-specific and m9p9-specific primers, with the primer containing the T7 promoter sequence that was used for in vitro transcription using the DIG RNA Labeling kit SP6/T7 (Roche): m9p9_5′-5′-GTGAAAGAAGTGCCACAGAA3′; m9p9_3′-5′-GGGCCCTAATAGCCTACTATAGTGCGGAGAAG3′; ftsH2_5′-5′-ATGAAATTTTCCTGGAGAACTG-3′; ftsH2_3′-5′-GGGCCCTAATAGCCTACTATAGTGCGGAGAAG3′). The RNA-RNA hybridization was performed for 16 h at 52°C and the signals were visualized by using Anti-Digoxigenin-AP FAB fragments and CSPD as substrate (Roche) and recorded by the LAS-1000 imager (Fuji). Signal intensity was estimated by using the PC-BAS program (Raytest). To exclude the unspecific binding of the ftsH2 probe to other ftsH mRNAs, we performed a control experiment using plasmid DNA containing each of the four ftsH genes. One microgram of plasmid DNA was blotted onto nitrocellulose membrane and used for hybridization with the ftsH2 probe. Only in the case of the plasmid with the cloned ftsH2 gene was a hybridization signal obtained, indicating the strict specificity of the used RNA probe (data not shown).

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