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Post-translational methylation of phycobilisomes and oxygen evolution efficiency in cyanobacteria

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A post-translationally methylated asparagine residue, γ-*N*-methylasparagine (NMA), is found at the β-72 site in many phycobiliproteins. We have examined the effects of asparagine methylation on photosynthetic rates in the wild-type cyanobacterium *Synechococcus* PCC 7942 (β-72 = NMA) and two *Synechococcus* PCC 7942 methylase mutants (β-72 = Asn) that exhibit no detectable methylase activity. Previous studies indicate that phycobilisomes isolated from non-methylated mutants exhibit a 14% decrease in fluorescence quantum yield (Swanson, R.V. and Glazer, A.N. (1990) J. Mol. Biol. 214, 787–796). Relative rates of Photosystem II electron transfer were measured for these strains by monitoring steady state rates of oxygen evolution in whole cells. The methylase-minus mutants demonstrated lower rates of electron transfer through Photosystem II under conditions in which the phycobilisome components were preferentially illuminated with orange light at low intensity. Oxygen evolution rates were indistinguishable for the wild-type and mutant strains upon selective illumination of the chlorophyll-containing light-harvesting antennae with blue light. The observed differences in photosynthetic rates are consonant with the levels of asparagine methylation in the phycobilisomes. These results support the hypothesis that asparagine methylation which is energetically costly yet evolutionarily conserved, is associated with increased energy transfer efficiency through Photosystem II.

Introduction

The light reactions of oxygenic photosynthesis begin with the trapping of radiant energy by auxiliary light-harvesting pigments. All photosynthetic organisms utilize some type of light-harvesting antenna system to maximize efficient energy collection by increasing the absorptive cross-section which energizes the reaction center [1]. Cyanobacteria and two groups of eukaryotic algae, the red algae and cryptomonads, contain light-harvesting antenna complexes composed of phycobiliproteins. These proteins can account for up to 50% of the light-harvesting capacity of these cells [2] and are arranged into macromolecular pigment-protein complexes known as phycobilisomes. The covalently bound linear tetrapyrrole (bilin) chromophores of the phycobiliproteins constitute 300–800 bilins in each phycobilisome aggregate and operate as a single unit. The phycobilisomes form regular arrays on the stromal side of the thylakoid membrane and function to absorb excitation energy in the range of 450–650 nm with energy transfer to the protein-chlorophyll complex of PS II [3,4]. The efficient directional transfer of excitation energy results from the protein spatial arrangement utilizing phycobiliprotein components which possess bilin prosthetic groups with differing absorption properties. Numerous structure/function studies have been performed on phycobiliproteins, including the determination of energy transfer rates within the phycobilisome [5–7] and spectroscopic studies of bilin chromophores [8,9]. In addition, an unusual post-translational modification, γ-*N*-methylasparagine, has been identified at the β subunit position 72 of many phycobiliproteins from prokaryotic cyanobacteria, eukaryotic red algae, and has most recently been found in the highly divergent eukaryotic cryptomonads [10–13].

The focus of our research is to elucidate the role of post-translational methylation in phycobiliproteins. Our experiments involve the use of two independently isolated asparagine methyltransferase mutants from *Synechococcus* PCC 7942 [14]. The mutant strains, designated *pcm*-1 and *pcm*-2, both produce allophycocyanin and C-phycocyanin that is unmethylated at position β-72. Phycobiliproteins isolated from these strains are indistinguishable in thermal stability and are expressed

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Abbreviations: DCBQ, 2,6-dichloro-*p*-benzoquinone; DMBQ, 2,5-dimethyl-*p*-benzoquinone; NMA, γ-*N*-methylasparagine; Chl, chlorophyll; PS II, Photosystem II.

in amounts comparable to the wild-type. C-Phycocyanin purified from the mutants exhibits a slight blue spectroscopic shift in its chromophore absorption. Isolated unmethylated phycobilisomes show fluorescence emission quantum yields that are 14% lower (recalculated from Ref. 14) than wild-type PCC 6301, representing diminished energy available from the phycobilisome terminal emitters. Purified phycobilisomes under these experimental conditions emit the excitation energy by fluorescence because they have been uncoupled from the membrane-bound PS II acceptors. This suggests that the β-72 methylation may be associated with increased energy transfer efficiency from phycobilisome components to the terminal energy acceptors in PS II.

In this report we have examined relative rates of PS II electron transfer for whole cells of *Synechococcus* PCC 7942 (wild-type), and the mutants *pcm*-1 and *pcm*-2. It was hypothesized that lower relative oxygen evolution efficiencies would be observed for the methylase-minus mutants but this difference was predicted to be no greater than 15%, based on the reported phycobilisome fluorescence emission data. Moreover, this advantage for methylated species should be manifested at low light intensity and disappear at saturating light where photosynthetic processes other than light-harvesting become limiting. In this communication we report oxygen evolution measurements for wild-type and methylase-minus strains of *Synechococcus* PCC 7942 and demonstrate that the observed differences in whole cell photosynthetic rates fit the predictions.

Materials and Methods

Strains and growth conditions

Synechococcus sp. strain PCC 7942 (Sherman) was obtained from Professor Louis Sherman, Purdue University; PCC 7942 (Berkeley), and the asparagine methylase-minus mutants *pcm*-1 and *pcm*-2 were obtained from Professor Alex Glazer, University of California, Berkeley. Cells were grown in BG-11 liquid media [15] at a constant temperature of 28–30°C in a shaking water bath with constant warm white light illumination of approx. 30 μmol photons/m^2 per s. Cells were harvested at mid-log phase (chlorophyll concentration $\sim$ 5–6 μg/ml), resuspended in fresh BG-11 and maintained at room temperature for subsequent oxygen evolution assays.

Oxygen evolution measurements

Steady state rates of oxygen evolution were measured by using a Clark-type electrode (Hansatech) that contained a water-jacketed 1 ml chamber maintained at 25°C. Air-saturated BG-11 medium was used for electrode calibration. The electrode was interfaced to a computer and oxygen evolution rates were stored every 5 s for a total assay period of 1.5 min. The data were analyzed by averaging the two highest consecutive 5 second rates for each light intensity. Illumination was provided by a 500 W xenon lamp alone or in conjunction with a broadband interference filter chosen to preferentially illuminate either the phycobilisomes (orange light) or chlorophyll (blue light). The phycobilisomes were selectively illuminated with a broadband interference filter (Oriel) with maximal transmission at 597.5 nm, and a bandwidth of 55 nm. Selective illumination of chlorophyll *a* was provided by an interference filter (Oriel) with a maximal transmission wavelength at 455.6 nm and a bandwidth of 73.2 nm. Maximal light intensities obtainable were approx. 6400, 1600 and 1500 μmol photons/m^2 per s for white, orange, and blue light, respectively. The data recorded in orange and white light as a function of light intensity were fit by non-linear regression analysis using the statistical analysis program ENZFITTER [16], assuming Michaelis-Menten kinetics.

Oxygen evolution in whole cells (10 μg chlorophyll [17]) was measured in BG-11 media in the presence of 1 mM artificial electron acceptors DCBQ or DMBQ (Eastman Kodak). This electron acceptor concentration yielded maximal rates of oxygen evolution for all four strains. Attempts to improve the assay including the addition of 1 or 5 mM $K_3Fe(CN)_6$ as a terminal electron acceptor in the presence of either DCBQ or DMBQ did not enhance oxygen evolution rates.

Quantum yield calculations were made by linear regression analysis forcing a line through the origin using the computer program INPLOT^{TM} (GraphPad Software) and normalizing the slope to that of PCC 7942 (Sherman) under identical light conditions. Combined data sets from the quantum yield measurements were subjected to a two-sided t-test for a 95% confidence interval using MINITAB statistical software (State College, PA).

Stoichiometry of asparagine methylation

Asparagine methylation was detected and quantitated by the presence of methylamine, the product of γ-*N*-methylasparagine hydrolysis, in acid hydrolyzates of purified phycobiliproteins. Cyanobacterial cells were labelled by growth in BG-11 containing 4 mM [*methyl*-^{3}H]methionine (100 mC$_i$/μmol) and the phycobiliproteins were purified [18]. Protein samples (2 nmol) were acid hydrolyzed for 24 h at 110°C in 6 M HCl containing 0.1% phenol and amino acid analysis was performed as described previously [19]. The radiolabelled hydrolyzates were characterized by collecting one minute fractions from the analyzer effluent followed by quantitation utilizing liquid scintillation counting. Stoichiometries were calculated assuming that C-phycocyanin and allophycocyanin contain three and five methionines, respectively, per [$\alpha\beta$] monomer.

Results and Discussion

Asparagine methylation

In order to study the effects of asparagine methylation on photosynthetic rates we have studied two independent nitrosoguanidine-induced mutants of *Synechococcus* PCC 7942 that are deficient in methylase activity. Mutants *pcm*-1 and *pcm*-2 are unmethylated at position β-72 in their phycobiliproteins as determined by methylamine analysis [14]. These mutants were derived from PCC 7942 (Berkeley) whose purified phycobiliproteins were found to be only partially methylated. This was surprising as this strain exhibited methylase activity comparable to that observed for PCC 6301 which is a fully methylated strain [14]. In contrast PCC 7942 (Sherman) which is maintained in our laboratory exhibits fully methylated phycobiliproteins. We have examined differences in methylation levels of these various strains and have obtained stoichiometries of methylation (Table I) that are similar to those reported for both *pcm*-1 and *pcm*-2. However, Swanson and Glazer [14] report a methylation level of 0.2–0.3 molar equivalents for PCC 7942 (Berkeley) C-phycocyanin, whereas our data indicate a level of 0.60; the origin of these discrepancies is unknown. The strain PCC 7942 (Sherman) which is fully methylated has been used for comparison purposes in this study.

Effect of asparagine methylation on photosynthetic rates

White light. The oxygen-evolving activity of whole cells containing fully methylated PCC 7942 (Sherman), hemi-methylated (PCC 7942, Berkeley), and unmethylated (*pcm*-1 and *pcm*-2) phycobiliproteins was monitored in whole cells using 1 mM DCBQ as an artificial electron acceptor. Light saturation curves were obtained in the intensity range of 25–3200 μmol photons/m^2 per s white light. The results suggest a trend of decreased oxygen evolution at low light that is related to the level of methylation in the phycobilisomes. PCC 7942 (Berkeley) and *pcm*-1 have relative oxygen evolution rates that are intermediate between PCC 7942 (Sherman) and *pcm*-2. PCC 7942 (Sherman) and *pcm*-2 were subsequently evaluated in greater detail. At low light intensities the mutant *pcm*-2 demonstrates photosynthetic rates that are 94% of the wild-type while *pcm*-1 reveals no difference as indicated by the relative quantum yield reported in Table II. When higher light intensities are employed the differences in oxygen evolution become less pronounced and this is marked by their similar average maximum observed velocities (554 and 526 μmol O_2/mg Chl per h for Sherman and *pcm*-2, respectively). This is expected when photosynthetic processes other than light harvesting become limiting at saturating light intensities. Kinetic analysis of these data (Table III) indicates that the strain containing fully methylated phycobilisomes exhibits a lower K_m than strains with diminished amounts of methylation.

TABLE I

Asparagine methylation of selected phycobiliproteins

Asparagine methylation was quantitated radiochemically by determining the ratio of methylamine to methionine in acid hydrolyzates of purified phycobiliproteins and converting this value to equivalents per [$\alpha\beta$] monomer.

Strain	Protein	Methylamine per [$\alpha\beta$]
PCC7942 (Berkeley)	C-phycocyanin	0.57
PCC7942 (Berkeley) *pcm*-1	C-phycocyanin	< 0.06 [a]
PCC7942 (Berkeley) *pcm*-2	C-phycocyanin	< 0.07 [a]
PCC7942 (Sherman)	C-phycocyanin	0.88
	allophycocyanin	1.0

[a] Experimental limit of detection.

TABLE II

Relative quantum yields of oxygen evolution

Relative quantum yields at low light intensities were calculated by linear regression analysis. Datasets are reported as an average ± standard deviation with the number of trials, N, in parentheses.

Experimental	PCC 7942	*pcm*-2	*pcm*-1
White light, DCBQ	1.0	0.94 ± 0.04 (2)	0.99 ± 0.04 (2)
Orange light, DCBQ	1.0	0.86 ± 0.06 (3) [a]	0.89 ± 0.01 (2) [a]
Orange light, DMBQ	1.0	0.91 ± 0.10 (4) [b]	
Blue light, DCBQ	1.0	1.01 ± 0.07 (2)	1.15 ± 0.26 (2)
Blue light, DMBQ	1.0	1.05 ± 0.05 (2)	

[a] Significantly different from PCC 7942 (Sherman) at the 99% confidence interval ($P < 0.01$).

[b] Not significantly different from PCC 7942 (Sherman) at the 95% confidence interval.

TABLE III

Kinetic parameters of oxygen evolution

Steady state rates of oxygen evolution in the presence of DCBQ were measured using a Clark-type electrode. Data are reported from a single experiment ± standard errors for the fitted parameters.

Experimental conditions	K_m (μmol photons/m^2 per s)	V_{max} (μmol O_2/mg Chl per h)
White light		
PCC 7942 (Sherman)	545 ± 34	781 ± 18
PCC 7942 (Berkeley)	623 ± 53	710 ± 23
PCC 7942 (Berkeley) *pcm*-1	611 ± 57	727 ± 25
PCC 7942 (Berkeley) *pcm*-2	659 ± 37	770 ± 15
Orange light		
PCC 7942 (Sherman)	363 ± 32	655 ± 23
PCC 7942 (Berkeley)	522 ± 27	776 ± 18
PCC 7942 (Berkeley) *pcm*-1	419 ± 32	678 ± 23
PCC 7942 (Berkeley) *pcm*-2	446 ± 28	760 ± 19

The observed differences in oxygen evolving activity in white light for *pcm*-2 do not prove that the differences are due to the level of methylation in the phycobilisome components. Because the methylase-minus mutants were generated by chemical mutagenesis it is possible that additional genetic lesions could be present. For this reason we also employed selective illumination conditions in our oxygen evolution assays. The following oxygen evolution experiments under different illumination conditions demonstrate that the observed differences in oxygen evolving capacity can be attributed directly to the phycobilisomes and not to any other components of the photosynthetic apparatus.

Orange light. The phycobilisome components were selectively illuminated with light at 598 nm. Oxygen-evolving activities which were measured under these conditions demonstrate a more profound difference between the fully methylated and unmethylated strains at lower light intensities. A light saturation curve for PCC 7942 (Sherman), *pcm*-1, and *pcm*-2 was obtained over the 0–1600 μmol photons/m^2 per s range. The relative quantum yield of oxygen evolution at low light intensities (0–100 μmol photons/m^2 per s) for *pcm*-2 is 86% of wild-type, as predicted by the reported fluorescence quantum yields [14]. This difference decreases as higher light intensities are achieved and is negligible at 200 μmol photons/m^2 per s. The average maximum observed oxygen evolution rates were 555 and 572 μmol O_2/mg Chl per h for Sherman and *pcm*-2, respectively. *Pcm*-1 displays a similarly diminished relative quantum yield of 89% in orange light. Kinetic analyses of these data are summarized in Table III. The Sherman strain displays the lowest K_m in the group and the Berkeley strain is higher, as expected. The mutant strains are intermediate between the two wild-type strains; this may be a consequence of other effects from the chemical mutagenesis. It is noteworthy that the quantum yield differences are expressed at levels well below the K_m so that the latter parameter is an insensitive measure of any differences.

Blue light. Chlorophyll *a* in both photosystems was preferentially illuminated by use of a broadband interference filter with a maximum transmission at 456 nm to demonstrate that decreased oxygen evolution in white light is not attributable to differences in the chlorophyll antenna. Illumination at 456 nm bypasses the phycobilisome components and provides a control. Under these experimental conditions oxygen evolution of the usual complement of fully, hemi-, or unmethylated strains demonstrates no significant difference in photosynthetic rates. The average maximum observed oxygen evolution rates were 147 and 134 μmol O_2/mg Chl per h for Sherman and *pcm*-2, respectively. However, fully saturating light intensities that would yield maximal rates of PS II electron transfer are not achievable under our experimental conditions with a practical limit of 1500 μmol photons/m^2 per s. This is not surprising because the chlorophyll antenna of PS II in cyanobacteria comprises only ~ 50 molecules and this absorption cross-section is small relative to a phycobilisome containing several hundred chromophores. Data analysis by curve-fitting to hyperbolic kinetics is not appropriate in this case because the maximal velocity is never approached and deviates from a rectangular hyperbola; better fits are found to a fourth order polynomial. Nonetheless, the differences in photosynthetic rates are unequivocally attributed to the phycobilisome components which are the location of NMA.

During the course of these experiments in blue light significant inhibition was observed presumably due to DCBQ photoreduction. Nedbal et al. [20] has observed a similar inhibition of photosynthetic rates with DCBQ in the presence of blue light. The nature of the inhibitor is unknown, however, its effect is pronounced in blue light; in orange light there was no measurable effect. An alternate electron acceptor, DMBQ, which did not demonstrate inhibition in the presence of blue light, was then chosen for the experiments. The average maximum observed oxygen evolution rates were 106 and 97 μmol O_2/mg Chl per h for Sherman and *pcm*-2, respectively in blue light using DMBQ. It was established that the maximum photosynthetic rates observed in the presence of DMBQ were approx. 35–40% lower than rates measured with DCBQ in both orange and blue light. Other investigators have observed a similar but more substantial decrease in oxygen evolution rates in the presence of DMBQ relative to DCBQ [20,21]. Despite diminished rates the data obtained in blue and orange light with DMBQ corroborated the patterns observed with DCBQ (Table II). However the orange light data were not statistically different in part because the lower overall rate contributes to a higher standard deviation for the rates.

Conclusions

Our results indicate that asparagine methylation in phycobiliproteins increases the photosynthetic efficiency demonstrated by steady state oxygen evolution techniques. The X-ray crystal structure of C-phycocyanin from *Mastigocladus laminosus* argues that NMA is less than 2 Å from the propionic acid side chain of ring B in the fluorescing chromophore which is responsible for interdisk transfer in the phycobilisome [22]. NMA is located in a turn between two helices and appears to shield ring B from solvent. The proximity of NMA suggests that methylation could alter the spectroscopic properties of the fluorescing chromophore [23]. Our data demonstrate that the level of asparagine methylation is congruent with increased photosynthetic rates under conditions where the phycobilisome components are preferentially illuminated. This trend of

lower relative photosynthetic rates for the methylase-minus mutants versus the wild-type PCC 7942 (Sherman) is observed at low light intensities where energy transfer through the light-harvesting complexes is expected to be limiting and disappears under saturating light conditions. Likewise, preferential illumination of chlorophyll *a* in both photosystems results in no demonstrable difference in photosynthetic rates between methylated and unmethylated strains utilizing either DCBQ or DMBQ. While the observed differences in oxygen evolution between the methylated and unmethylated strains are small, they are consistent with the 14% differences in phycobilisome fluorescence quantum yield originally reported [14]. Thus methylation can improve the PS II quantum efficiency from 0.82 to 0.95 (the generally accepted value for phycobilisome energy transfer efficiency [3,7]).

Presumably such a difference provides a selective advantage to cells growing in aquatic niches which are light-limited. Laboratory and field experiments with marine *Synechococcus* sp. indicate that natural light levels for oceanic cyanobacteria are commonly in the 30–200 μmol photons/m^2 per s range [24,25]. This contrasts with illumination on the earth's surface which is $\sim$2000 μmol photons/m^2 per s at mid-day [3]. Aquatic euphotic zones commonly extend to levels near 1% of the surface solar radiation. The optical properties of water dictate that physiological ecology of phytoplankton include adaptations to orange light at low intensity.

These results suggest an interesting structure/function relationship for asparagine post-translational modifications in proteins. This modification is not known to be present in any other proteins but is highly conserved in cyanobacteria and red algae. Asparagine methylation of proteins is now one of the few stable post-translational methylations for which a function can be demonstrated. The function of arginine, histidine, and lysine methylation in proteins remains controversial [26].

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