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Chlorophyll Fluorescence Analysis of Cyanobacterial Photosynthesis and Acclimation

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INTRODUCTION

Principles of Modulated Fluorescence Analysis

Chlorophyll fluorescence analysis allows noninvasive, near-instantaneous measurement of key aspects of photosynthetic light capture and electron transport. For natural samples, fluorescence signals are specific to photobionts and allow in situ measurements of small (61) or dilute (65, 130) mixed natural populations. For molecular studies, fluorescence signals can be used for rapid screening of mutant or transgenic colonies and cultures and for tracking physiological processes during gene regulation experiments. Rapid screening has become increasingly important with the advent of genomic sequencing and saturation mutagenesis. Therefore, applications of chlorophyll fluorescence are expanding in both field and laboratory settings.

In cyanobacteria, the photosynthetic system is tightly connected to the other principal metabolic paths and is in itself a major metabolic sink for iron, nitrogen, and carbon skeletons. Therefore, chlorophyll fluorescence signals can provide rapid, real-time information on both photosynthesis and the overall acclimation status of cyanobacteria. We and other groups have been adapting to cyanobacteria techniques of in vivo fluorescence analysis originally developed for plants (5, 20–28, 58, 61, 69, 74, 80, 87–91, 112, 116, 128, 133, 138, 141, 142, 150).

Fluorescence analysis depends on the phenomenon that when a pigment absorbs the energy of a photon and enters an excited electronic state, there are essentially four routes for the return to ground state: (i) photochemical reactions in which the excited electron leaves the pigment molecule and enters an electron transport chain, as occur in specific chlorophylls in photosynthetic reaction centers; (ii) heat dissipation, in which the excited electron returns to ground state by releasing heat; (iii) transfer of the excitation energy to an adjacent pigment, as occurs in the light-harvesting antenna systems of photosynthetic organisms; and (iv) emission of a fluorescence photon, of a wavelength longer than that of the photon initially absorbed. These four processes are in competition, and for a given excited molecule, the path with the largest first-order rate constant predominates. For biological systems, the overall chlorophyll fluorescence yield is usually low, and in vivo chlorophyll fluorescence from photosystem II (PS II) predominates (38, 66, 112). In cyanobacteria, phycobiliproteins also contribute fluorescence, which overlaps with the spectrum of chlorophyll emission.

Although PS II fluorescence is a minor pathway for excitation dissipation, it competes with the quantitatively more important energy dissipation routes of PS II photochemistry, exciton transfer to other pigment systems (such as PS I), and heat dissipation. Therefore, changes in photochemistry or in

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TABLE 1. Fluorescence levels and associated light treatments used for cyanobacterial fluorescence quenching analysis[a]

Fluorescence level	Light treatment (weak modulated measuring beam throughout)
F_O	Dark
$F_{M\text{dark}}'$	Dark + saturating flash
F_S	Actinic light
F_O'	Dark or weak far-red
F_M'	Actinic light + saturating flash
F_M	Actinic light + DCMU

[a] For further details see Appendix.

the two nonphotochemical routes (energy transfer and heat emission) cause changes in the fluorescence yield from PS II (13, 66, 112). When the potentials for photochemistry and nonphotochemical dissipation are minimal, the fluorescence yield is maximal. Quenching or lowering of the fluorescence yield below its maximum occurs when excitation flow increases to the competing photochemical or nonphotochemical pathways.

To deduce information on photosynthesis from analysis of fluorescence quenching, one assumes that changes in fluorescence yield reflect proportional changes in the competing deexcitation pathways of photochemistry, exciton transfer, and heat dissipation. This basic assumption is not strictly valid (53, 56, 144). Nevertheless, the fluorescence signal is rich in information, and in plants the parameters F_V/F_M, F_V'/F_M', q_P, q_N, NPQ, and ϕPS II are empirically verifiable indices of photosynthetic performance and acclimation status (12, 36, 45, 66, 67, 82, 99, 102–104, 106, 107, 127, 129, 134, 145, 153).

For any pigment, the level of fluorescence emission depends on the pigment concentration, the excitation light intensity, and the fluorescence yield or efficiency of fluorescence emission. For fluorescence quenching analysis, the excitation intensity and pigment concentration must be constant, so that changes in fluorescence reflect the changes in fluorescence yield which result from the competing photochemical and nonphotochemical deexcitation pathways. Changes in pigment concentration are generally not a concern over the brief periods of fluorescence measurements.

Modulated fluorometers are currently widely used to measure in vivo chlorophyll fluorescence from plants and increasingly from cyanobacteria in both the laboratory and the field (128, 130). This review concentrates on data obtained with modulated fluorometers, although other approaches are also used (40). Modulated fluorometers specifically detect and amplify only the fluorescence excited by a weak, constant measuring beam consisting of a train of low light pulses at a frequency of 1 to 100 kHz. Therefore, the excitation intensity is constant and changes in the fluorometer signal reflect changes in fluorescence yield. The modulated measuring beam is sufficiently weak that it drives essentially no photosynthesis, allowing determination of the fluorescence yield of dark-adapted samples. Furthermore, since the detection system ignores fluorescence excited by other light, it is possible to change the actinic light and provide multiple saturating pulses of light over the course of one measurement (Table 1; see Fig. 2). The fluorescence yield can therefore be measured under different levels of actinic light, and saturating flashes can be used at any point to momentarily close all PS II centers and drive photochemical quenching to zero (see Fig. 2).

Goals and Scope

In this review, we discuss pulse-amplitude modulated fluorescence as a rapid, noninvasive monitor of acclimation and photosynthesis in cyanobacteria and cyanolichens. We do not cover the biophysical mechanisms underlying chlorophyll fluorescence emission, which are well reviewed elsewhere (31, 38, 66, 112, 127, 137, 141, 144). Rather, we summarize some of the potentials and limitations of fluorescence analysis for extracting physiologically and ecologically useful information from cyanobacteria, whose photosynthetic physiology (see Fig. 1) and fluorescence patterns (see Fig. 2) differ in important respects from those of plants (20, 22, 91, 112, 128, 141). In particular, we demonstrate how characteristic changes in nonphotochemical quenching of fluorescence can be used to estimate the light level to which the sample is acclimated. This information can then be used in conjunction with the ϕPS II parameter to estimate electron transport under acclimated conditions.

INTERPRETING CYANOBACTERIAL FLUORESCENCE SIGNALS

A Distinct Photosynthetic System Yields Distinct Fluorescence Signals

The central PS II and PS I photosynthetic complexes are very similar in plants and cyanobacteria, as are many elements of the light capture, electron transport, and carbon dioxide fixation systems. Nevertheless, cyanobacteria are metabolically flexible prokaryotic organisms, with several key structural and metabolic distinctions which strongly influence the nature and interpretation of their fluorescence signals (Fig. 1).

In cyanobacteria, the principal light-harvesting complexes are phycobilisomes peripheral to the thylakoid membranes, rather than the integral membrane chlorophyll-*a*/*b* binding proteins which capture light in plants. Cyanobacterial phycobilisomes diffuse along the surface of the thylakoids, at a rate sufficient to allow movement from PS II to PS I within 100 ms (96). This distinction in light capture structures between plants and cyanobacteria has many metabolic and functional consequences (7). In particular, the cellular phycobiliprotein content influences cellular fluorescence yield. Furthermore, cyanobacteria have high and variable ratios between PS I and PS II complexes (98, 112), so that in comparison with plants, PS II accounts for relatively little of the cellular chlorophyll. This can also influence the interpretation of fluorescence signals, since the variable fluorescence component arises from PS II while the constant or F_0 fluorescence component contains emissions from PS II, phycobiliproteins, and possibly also PS I chlorophyll (112).

Photosynthetic and respiratory electron flow both occur in cyanobacterial thylakoid membranes (62, 121), sometimes simultaneously, and they share numerous electron transport intermediates (Fig. 1). Under illumination, there is net input of electrons into the transport system from the water-splitting activity of PS II. Under light or dark conditions, there are variable electron fluxes from NAD(P)H, which is oxidized by one or more thylakoid-bound dehydrogenases (11, 54, 85, 86, 121, 139). Electrons from ferredoxin can also enter the transport system, possibly passing via the same complex(es) that catalyze NAD(P)H oxidation (86). In a photoautotroph, electrons derived from NAD(P)H or ferredoxin are clearly not a net input of reductant into the system; rather, they represent some form of cyclic flow, since the electrons used to reduce NAD(P)H or ferredoxin derive ultimately from the water-

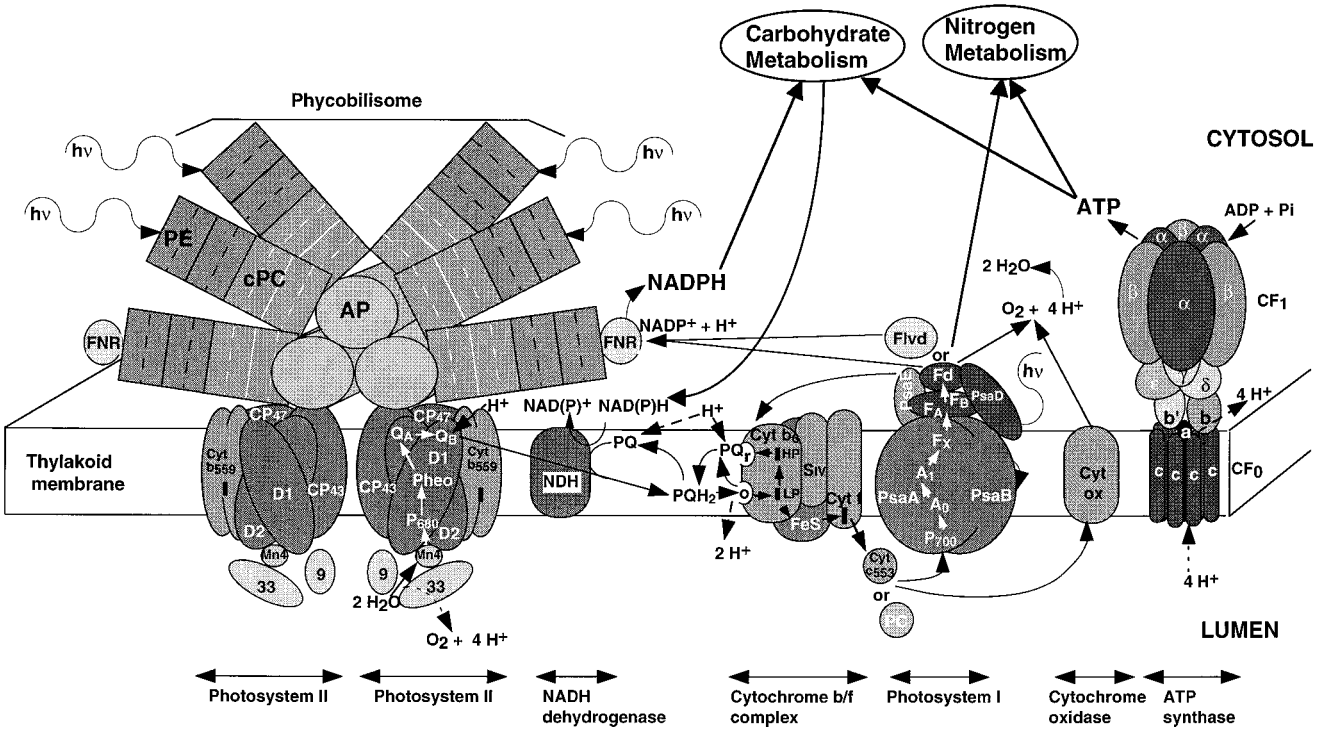

FIG. 1. Cyanobacterial thylakoid electron transport. This schematic diagram is based on data primarily from *Synechocystis* sp. strain PCC 6701, which contains phycoerythrin, phycocyanin, and allophycocyanin pigment proteins in the phycobilisomes. The phycobilisomes move rapidly along the surface of the thylakoid membranes (96), so the phycobilisome-PS II dimer complex is transient. Excitation absorbed by the phycobilisome can reach either PS II or PS I, particularly in cells in state II. This excitation flow may involve specialized subunits of the phycobilisome core (not shown here). The composition and organization of the phycobilisome rods and core is variable in different cyanobacteria; the three-cylinder core and six-peripheral rod configuration is common, but in *Synechococcus* sp. strain PCC 7942 the core contains only two cylinders. The PS I-PS II stoichiometry is usually higher than 1:1; in the *Synechococcus* cells used for most experiments described in later figures, the ratio was 2 to 3 PS I/PS II. There are multiple interacting and flexible paths of electron flow including linear flow from water to NADPH; several possible cyclic pathways around subsections of the transport system; pseudocyclic flows from water with electron donation back to oxygen; and respiratory flows of electrons derived from reserve molecules. Some possible flows are indicated by black arrows. The donor-acceptor stoichiometries of electron transfers are not shown, but various redox centres carry different numbers of electrons, from 1 (e.g., PC, cytochrome c_{553}, Fd, and Flvd), 2 [e.g., PQ and NAD(P)H] and even 4 (the Mn complex of PS II). The redox reactions are reversible depending upon the oxidation-reduction status of the acceptor-donor pair and the local proton concentration, so that in some cases the indicated direction of electron flow could be reversed. Proton uptake and transmembrane transport are indicated by dashed arrows; other proton translocation pathways may also exist. The plastoquinone-plastoquinol pool can be reduced by electrons from PS II, from an NAD(P)H dehydrogenase(s) (NDH) whose composition and substrate specifities vary between strains, and from the "r" site of the cytochrome *bf* complex. Plastoquinone reduction by NDH is the entry point for electrons derived from respiration. The various plastoquinone reductions involve proton uptake from the stroma, while oxidation of a plastoquinol at the 'o' site of the cytochrome *bf* complex releases two protons to the lumen. As indicated, electron and ATP flow to carbohydrate and nitrogen metabolism can have strong and rapid effects on thylakoid function, while electrons derived from carbohydrate reserves also enter the thylakoid system and influence photosynthetic function. During ATP synthesis, protons enter a channel from the lumen formed by the a subunit of Cf_0, and their exit to the cytosol is coupled to rotation of the ring of c subunits through directed diffusion (37). The c-ring rotation drives rotation of the γ subunit of CF_1 within the $\alpha_3\beta_3$ ring (117), which in turn drives a sequence of conformational changes in three identical ATP/ADP binding sites. The changes in binding site lead to phosphorylation of ADP and expulsion of ATP from the site. The stoichiometry is 1 ATP/4H^+ passing through the complex (146). Abbreviations: hν, photons of visible light; PE, phycoerythrin $\alpha_3\beta_3$ trimers; CPC, phycocyanin $\alpha_3\beta_3$ trimers; AP, allophycocyanin rods of the phycobilisome core, composed partly of $\alpha_3\beta_3$ trimer disks along with other related phycobilin-binding proteins; D1 and D2, core polypeptide dimer of PS II which binds the redox cofactors; Cyt b_{559}, cytochrome b_{559} in the PS II core; Mn4, manganese cluster of the oxygen evolving complex; 9 and 33, 9- and 33-kDa subunits of the oxygen evolving complex of PS II; CP43 and CP47, 43- and 47-kDa chlorophyll protein complexes associated with the PS II core; P_{680}, dimeric chlorophyll center which is photooxidized in PS II; Pheo, pheophytin primary electron acceptor of PS II; Q_A, the quinone secondary electron acceptor of PS II; Q_B, a plastoquinone bound to PS II which accepts two electrons from Q_A and equilibrates with the thylakoidal plastoquinone-plastoquinol pool; NDH, NAD(P)H dehydrogenase (in various strains there are different forms of the complex with differing activities and specificities for NADH or NADPH); Cyt b_6, a cytochrome containing both low- and high-potential heme centers which are involved in a Q-cycle electron flow from plastoquinol bound to the o site to plastoquinone bound to the "r" site (this cycle results in proton translocation); S_{IV}, subunit IV of the cytochrome *bf* complex; FeS, an iron-sulfur redox center; PC, plastocyanin, a copper-containing luminal single-electron transport protein; Cyt c_{553}, cytochrome c_{553}, a heme-containing luminal single-electron transport protein (plastocyanin and cytochrome c_{553} can be reciprocally regulated in response to copper and iron availability); PsaA and PsaB, related chlorophyll binding proteins which form the core of PS I; P_{700}, the chlorophyll which is photooxidized in PS I; A_0, A_1, F_X, F_A, F_B, bound redox intermediates of PS I; Flvd, flavodoxin (a flavin protein which is a cytosolic mobile single electron carrier that can accept electrons from PS I and that can substitute for ferredoxin, particularly under low-iron conditions); Fd, ferredoxin (an iron protein which is a cytosolic mobile single-electron carrier that can accept electrons from PS I and can transfer the electrons to NADPH or participate directly in some biosynthetic reactions, particularly in nitrogen metabolism); FNR, ferredoxin/flavodoxin NADPH oxidoreductase; Cyt ox, the cytochrome oxidase complex involved in respiratory electron transport (it can also withdraw electrons from photosynthetic electron transport, particularly under excess light); α to γ, subunits of the CF_1 complex of ATP synthase; a to c, subunits of the CF_0 complex of ATP synthase. 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splitting activity of PS II. Typically, cyclic electron flow is used to describe flow from PS I via ferredoxin and/or NADPH (54, 86) directly back to the intersystem transport chain. This cyclic flow can drive proton translocation through localized reductions of plastoquinone at the cytosolic side of the thylakoid, with concomitant proton uptake, and plastoquinol oxidation near the luminal side, with proton release to the lumen (55) (Fig. 1). More generally, electrons derived from the oxidation of carbohydrates or other reserve molecules can be carried by NAD(P)H into the thylakoid intersystem transport chain via the thylakoid-bound dehydrogenase(s). These electrons from the reserve molecules are derived ultimately from PS II water

splitting and, upon reentry to the thylakoid system, can pass to oxygen under light or dark or to PS I (139) under illumination. The reserves thus act as an electron bank so that the flow into the thylakoid system can be offset in time from the original photosynthetic production of reductant, with important regulatory consequences (34, 84, 94).

In all known cases, electrons from these various inputs come together at the cytochrome *bf* complex, which is a plastoquinol oxidoreductase (68) (Fig. 1). There are two plastoquinone binding sites in the complex, which allow for a Q cycle, in which some of the electrons removed from plastoquinol at the luminal side of the membrane are cycled within the complex and passed back to plastoquinone bound near the cytoplasmic side of the membrane. This branch of the transport chain allows the cyanobacteria to increase the number of protons translocated per net electron passing through the transport chain. Since the reduction involves proton uptake from the cytosol and the oxidation releases protons to the lumen, electron flux through the Q cycle must respond sensitively to the magnitude of the proton gradient across the membrane.

The primary electron flux through the cytochrome *bf* complex is from plastoquinone to luminal electron carriers, primarily plastocyanin or cytochrome c_{553} (81, 113), which transport the electrons either to PS I or to a cytochrome oxidase complex which may include cytochrome *c*(m) (81). Plastocyanin and cytochrome c_{553} can each fulfill transport roles to PS I or the cytochrome oxidase (81). Double-inactivation mutants mutated in both proteins are inviable in some (81) but not all (155) strains, so that some strains must have an alternate route for electron flow away from cytochrome *bf*. Although single-inactivation mutants mutated in one or the other protein are viable (26, 71, 81), the loss of one protein can lower the capacity for electron flux away from PS II, particularly under conditions of excess excitation (26). Conversely, overexpression of heterologous plastocyanin in *Synechococcus* can increase the electron transport capacity (44). Thus, although partially complementary, the two proteins may play somewhat distinct functional roles. Furthermore, plastocyanin contains a copper redox cofactor while cytochrome c_{553} contains an iron redox cofactor, and in some strains they are differentially regulated in response to copper and iron availability (15, 16, 119, 120, 154). Another example of alternate electron carriers is the iron-sulfur protein ferredoxin, which accepts electrons from PS I but which can be replaced under conditions of iron stress by flavodoxin (70, 75).

The flow of electrons to oxygen as a final acceptor responds rapidly to environmental and metabolic conditions and can be an important element in preventing overreduction of PS II and the intersystem transport chain under excess illumination (22a, 88, 147). This flow to oxygen can be mediated by cytochrome oxidase activity (131) or by photoreduction of oxygen by electrons from PS I, either directly (3, 79) or via ferredoxin (43, 50).

Carbon metabolism and nitrogen metabolism in the prokaryotic cyanobacteria occur in close proximity to the cytosolic surface of the thylakoids and so have strong and direct influences on electron transport and hence on fluorescence (63, 83, 84, 92, 116, 122), both as sinks for ATP and electrons and as sources of electrons extracted from reserve molecules.

In summary, this system forms a web of electron sources and sinks, linked by interconnected redox intermediates, that allows for flexible and rapid shifts in electron fluxes in response to environmental or metabolic changes (5, 54, 55, 74, 80, 81, 85, 86, 131, 135, 147). Furthermore, through shared electron transport carriers, respiration directly influences the photosynthetic regulatory status and vice versa (34, 84, 94). Several of the characteristic properties of cyanobacterial fluorescence signals result from these respiration/photosynthesis interactions, including their distinct patterns of photochemical and nonphotochemical quenching.

In plants, a cycle of conversions of xanthophyll carotenoids is driven by the *trans*-thylakoid ΔpH gradient and is implicated in regulating nonphotochemical dissipation of excess light energy (1, 46, 47, 57). Cyanobacteria lack this cycle (33) but have alternate strategies to cope with excess excitation (105, 147). Finally, cyanobacteria show changes in the functional organization of the light capture system, termed state transitions, which can result in large changes in the PS II fluorescence yield depending upon the level of illumination (7, 14, 97, 111, 112). In contrast, in higher plants, state transitions have relatively minor influences on PS II fluorescence (66). This review deals now with how the distinct organization and function of cyanobacterial photosynthesis lead to opportunities and limitations for chlorophyll fluorescence analysis of cyanobacteria.

F_O, F_V/F_M, and F_O' in Cyanobacteria versus Plants

Ting and Owens (141) have shown that for any chlorophyll-containing suspension, the values of F_O, F_M and F_V/F_M measured with a modulated fluorometer vary somewhat with pigment concentration. Therefore, within a set of experiments, the chlorophyll concentration should be standardized if precision is required or, alternately, a small correction could be introduced to compensate for variation in the chlorophyll concentration. In our experiments, chlorophyll concentrations from 1 to 3 μg/ml gave results sufficiently consistent for quenching analysis. The use of alternate cuvettes and detectors can greatly extend this concentration range (125).

A more fundamental problem with the measurement of F_O in cyanobacteria is that F_O fluorescence varies considerably depending on the cellular phycobiliprotein concentration. Figure 3 illustrates that as the phycocyanin/chlorophyll ratio of wild-type *Synechococcus* sp. strain PCC 7942 rises, F_O fluorescence also increases, particularly once the phycocyanin content is increased above a threshold level. This phycobiliprotein contribution to F_O fluorescence is not influenced by changes in the redox state of PS II (51, 72, 111). It could be a low-yield fluorescence emission from coupled phycobilisomes or a high-yield emission from a small population of uncoupled phycobilisomes (96) or free phycobiliproteins. The exact source(s) of this phycobiliprotein fluorescence could be addressed by studies using cyanobacteria with a range of phycobiliprotein contents analyzed with the range of different color light-emitting diodes now available as modulated fluorescence excitation sources (65). In a *Synechococcus* sp. strain PCC 7942 mutant lacking phycocyanin, F_O is low (Fig. 3), which confirms the F_O/phycobiliprotein correlation observed in the wild type. Furthermore, in this mutant strain, F_V/F_M under acclimated growth is about 0.75 (157), as opposed to values of 0.4 to 0.6, typical of wild-type *Synechococcus* grown under the same conditions.

In plants F_V/F_M $[(F_M - F_O)/F_M]$ (Fig. 2) is well verified as an index of the maximal photochemical efficiency of PS II (12), but this interpretation depends on both F_O and F_V originating predominantly from PS II. This assumption is not valid for cyanobacteria (20, 111, 112, 128), since phycobiliprotein fluorescence contributes to F_O and PS II accounts for only a small proportion of total chlorophyll. In higher plants under ideal conditions, F_V/F_M is near 0.8 and lower values reflect inhibition of PS II function (12). In cyanobacteria, changes in F_V/F_M under conditions of constant pigment content correlate well with changes in independent measurements of PS II function

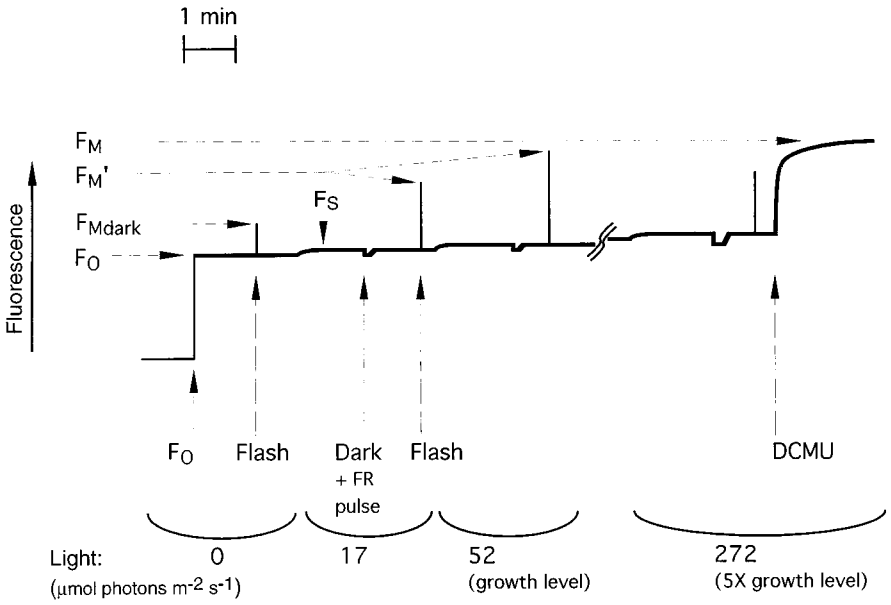

FIG. 2. Fluorescence emission trace for cyanobacterial quenching analysis. This trace from *Synechococcus* sp. strain PCC 7942 shows a typical cyanobacterial response over a series of increasing light intensities. The brief pulses of saturating light result in a rapid increase in fluorescence as PS II centers close transiently. The measurement terminates with addition of DCMU, which closes PS II centers, causing a rapid rise in fluorescence followed by a slower fluorescence rise phase as the cells go to full state I. Modified from reference 23 with permission of the publisher.

such as oxygen evolution (24, 27, 28, 80), but the absolute level of F_V/F_M is not a reliable indicator of PS II function. The distortion of F_O fluorescence is pronounced only at high cellular concentrations of phycocyanin (Fig. 3), which may be achieved primarily under nutrient-rich artificial culture conditions, as used in our experiments to date. When interpreted with caution, F_V/F_M is still a useful parameter, particularly if the same sample is monitored repeatedly over time and if the cellular pigment content is constant. As expected, the prochlorophyte *Prochloron* shows a high value of F_V/F_M (126), consistent with the lack of phycobilisomes and the chlorophyll-based antenna system in this group of cyanobacterial relatives.

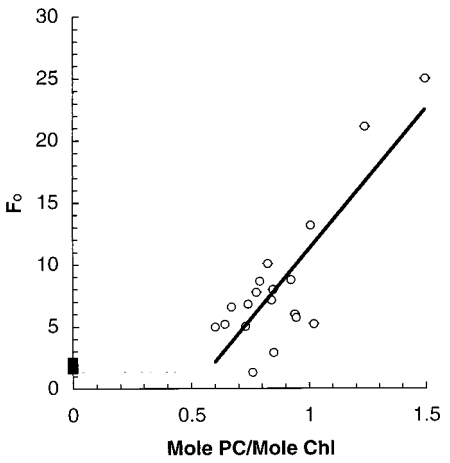

FIG. 3. F_O fluorescence increases with the phycocyanin content in *Synechococcus* sp. strain PCC 7942. F_O fluorescence is normalized to the molar chlorophyll concentration to allow for different culture concentrations and plotted against the molar ratio of phycocyanin (PC) to chlorophyll (Chl). Each point is a single determination on an independently grown culture. ○, wild-type cells; ■, mutant strain which lacks phycobilisome rods and contains no phycocyanin. Note that at low phycocyanin contents, F_O in the wild type falls toward the level in the rodless mutant. Modified from reference 23 with permission of the publisher.

F_O' is the minimal fluorescence level with all PS II reaction centers open. It is measured with cells under a given light acclimation status but transferred briefly to darkness or far-red light. The determination of F_O' is a problematic aspect of quenching analysis in cyanobacteria, since under moderate light intensities F_O' is often very close to the steady-state F_S fluorescence level (Fig. 2). Furthermore, unlike in plants, F_O' in cyanobacteria is usually higher than the F_O fluorescence as a result of the dark-to-light increase in PS II fluorescence yield, i.e., the state transition (see below). It is therefore difficult to distinguish the initial small but rapid drop in fluorescence yield as PS II centers open, from the slower state transition-dependent decline to the dark F_O fluorescence level. Computerized data logging might alleviate this problem by resolving the fluorescence relaxation kinetic phases.

In higher plants, F_V'/F_M', defined as $(F_M' - F_O')/F_M'$ (Fig. 2), reflects the photochemical efficiency of open PS II centers under a given light acclimation status (45). F_V'/F_M' generally varies inversely with q_N (see below), since nonphotochemical energy dissipation lowers the photochemical efficiency of PS II below the maximum levels reflected by F_V/F_M. A drop in F_V/F_M, as occurs during photoinhibition of PS II activity, also feeds through and results in a drop in F_V'/F_M'. Thus, in a plant, changes in the F_V'/F_M' parameter reflect the combined regulation of PS II through both reversible nonphotochemical quenching and photoinhibitory inactivation of PS II.

In cyanobacteria, changes in F_V'/F_M' also combine nonphotochemical influences on PS II function and photoinhibitory inactivation of PS II. As described below, nonphotochemical quenching of cyanobacterial PS II fluorescence results primarily from changes in excitation distribution between the two photosystems rather than from excitation dissipation. Therefore, in a cyanobacterium, a drop in F_V'/F_M' can result from a photoinhibitory drop in F_V/F_M or from a regulatory redistribu-

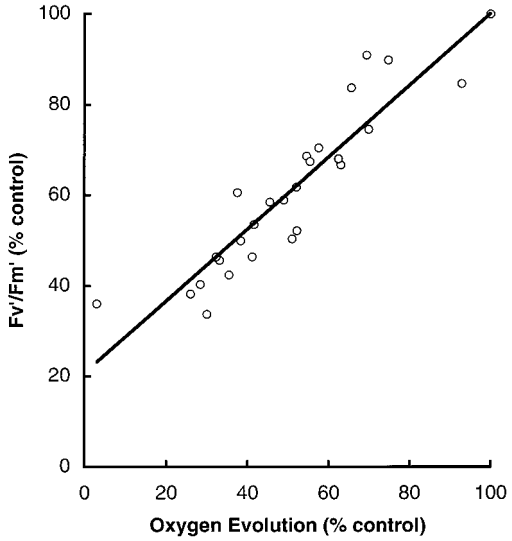

FIG. 4. Photoinhibition of oxygen evolution correlates with declines in F_V'/F_M' in *Synechococcus* sp. strain PCC 7942. Cultures were subjected to a photoinhibitory decrease in growth temperature from 37 to 25°C. Oxygen evolution and F_V'/F_M' were monitored and expressed as a percentages of the pretreatment control values. Four cultures were used. $y = 0.79x + 21$; $R^2 = 0.89$.

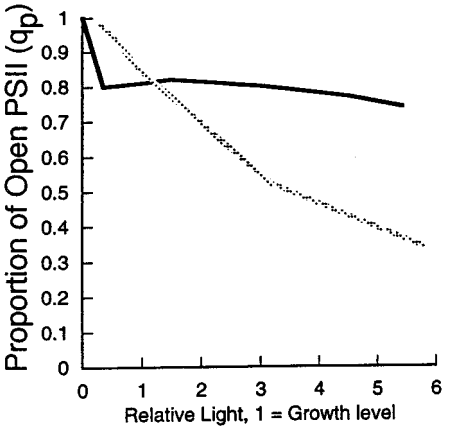

FIG. 5. Photochemical quenching of fluorescence plotted against light intensity. Typical light response curves of photochemical quenching in a cyanobacterium, *Synechococcus* sp. strain) PCC 7942 (solid line), and in rye for a plant-type pattern (dotted line) are shown. For comparison, light intensity is expressed relative to the growth light intensity; 1 = growth level. The actual growth light intensities were 50 μmol of photons m^{-2} s^{-1} for the cyanobacterium and 250 μmol of photons m^{-2} s^{-1} for the rye.

tion of excitation from PS II to PS I. In plant fluorescence analysis, a common implicit assumption is that down-regulation of PS II reflects overall down-regulation of photosynthetic electron transport. This assumption is not applicable to cyanobacteria, which have more flexible excitation distribution and electron transport systems. Furthermore, cyanobacterial F_V'/F_M' suffers the same limitations as described above for F_V/F_M, which are further compounded by the difficulty of measuring the F_O' fluorescence level. Nevertheless, as shown in Fig. 4, changes in measured F_V'/F_M' correlate well with changes in oxygen evolution during a photoinhibitory treatment (24, 27). Cyanobacterial F_V'/F_M' is a useful integrated measure of PS II activity, even though various mechanisms may underlie the changes in PS II function.

PHOTOCHEMICAL QUENCHING AND EXCITATION PRESSURE

Photochemical and nonphotochemical quenching measure changes in variable fluorescence, which derives from PS II; they are therefore less susceptible to distortion from non-PS II contributions to F_O fluorescence. They involve minimal mechanistic assumptions (145), although the terms q_P and q_N often carry mechanistic associations which are not applicable to cyanobacteria.

Photochemical quenching reflects a lowering of fluorescence below maximal levels through photochemical competition with fluorescence emission. Thus, when all PS II reaction centers are open and the potential for photochemistry is maximal, photochemical quenching of fluorescence is also maximal and fluorescence yield is low (F_O or F_O'). Conversely, when all PS II centers are closed and no photochemistry can occur, photochemical quenching is zero and the fluorescence yield is maximal (F_M or F_M'). In practice, photochemical quenching is quantified by the photochemical quenching coefficient (145):

$$q_P = (F_M' - F_S)/(F_M' - F_O')$$

This parameter gives the position of steady-state fluorescence, F_S, on the scale from F_O' (all PS II centers open) to F_M' (all PS II centers closed) (Fig. 2). Thus, if steady-state fluorescence is equal to F_O', $q_P = 1$, indicating that all PS II centers are open. If steady-state fluorescence equals F_M', $q_P = 0$, indicating that all PS II centers are closed. Between these extremes, progressive reaction center closure is reflected by a declining q_P, although the relationship is not strictly linear (20) because of excitation migration from closed to open reaction centers (66) and fluorescence quenching by oxidized plastoquinone (148). The absolute level of F_O or F_O' does not distort the calculation, since it is scaled to variable fluorescence, $F_V' = F_M' - F_O'$, under a given condition.

q_P reflects the balance between excitation of PS II centers, which closes them, and removal of electrons from PS II by the electron transport chain, which reopens the centers. This balance, or excitation pressure on PS II, responds not only to incident light intensity (27) but also to factors influencing electron flow away from PS II, such as temperature (24, 59, 82, 101, 102, 106, 107) and the availability of terminal electron acceptors such as CO_2 or O_2 (88, 147). Indeed, the pivotal position of PS II in photosynthetic electron transport means that environmental and metabolic signals are integrated into q_P, which is thus a general index of the balance between energy capture and consumption.

As shown in Fig. 5, in cyanobacteria and cyanolichens, q_P typically stays high over a broad range of incident light intensity, up to 10 times higher than the growth light intensity (21, 23, 27, 28, 80, 138). This contrasts sharply with the pattern typical of higher plants, where q_P falls progressively as the light intensity exceeds the growth light. This cyanobacterial capacity to maintain PS II centers open under excess light reflects a complex and flexible electron transport system (5, 44, 55, 85, 86, 131, 135), as well as a generally high PS I/PS II ratio (23, 42, 98, 112). In particular, cyanobacteria have a very high and flexible capacity to remove electrons from PS II, with oxygen as a terminal acceptor for electron flow from water (21a, 88, 91, 128, 147). This flow is low under low light, variable but significant at the growth light intensity, and large under excess light (see below). It thus serves to buffer PS II from excess excitation by removing electrons as required. At least part of this flow is

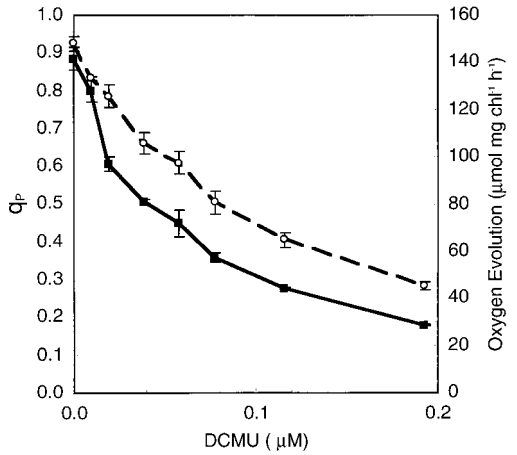

FIG. 6. Photochemical quenching of fluorescence under DCMU treatment compared with oxygen evolution in *Synechococcus* sp. strain PCC 7942. DCMU inhibits oxygen evolution (○), closing PS II centers in parallel (q_P) (■). Results are means and standard errors of measurements on the same culture at 37°C (n = 3). Modified from reference 24 with permission of the publisher.

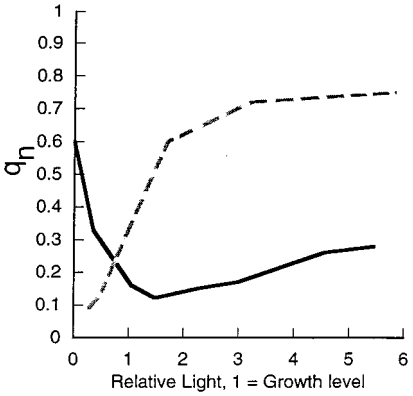

FIG. 7. Nonphotochemical quenching of fluorescence plotted against light. Typical light response curves of nonphotochemical quenching in a cyanobacterium, *Synechococcus* sp. strain PCC 7942 (solid line), and in rye for a plant-type pattern (dotted line) are shown. For comparison, light intensity is expressed relative to the growth light intensity; 1 = growth level. The actual growth light intensities were 50 μmol of photons m^{-2} s^{-1} for the cyanobacterium and 250 μmol of photons m^{-2} s^{-1} for the rye.

sensitive to cyanide inhibition, suggesting a contribution from respiratory electron flow through cytochrome oxidase (131).

In spite of the different patterns of q_P in cyanobacteria and plants, the parameter does successfully measure PS II closure in cyanobacteria, as shown by titration with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). This inhibitor binds to the Q_B binding site of PS II, blocking electron flow and causing reaction center closure. Figure 6 shows that in *Synechococcus* sp. strain PCC 7942, as expected, q_P and oxygen evolution drop in parallel upon progressive DCMU inhibition of PS II. Furthermore, our work on *Synechococcus* sp. strain PCC 7942 showed that although this strain has a strong capacity to maintain PS II open, even fractional closure of the reaction centers can lead to photoinhibition and large changes in gene expression, indicative of active acclimation processes (24, 27, 28) in response to relatively small drops in the proportion of open reaction centers.

PREDICTING LIGHT ACCLIMATION STATUS

State Transitions Dominate Nonphotochemical Quenching of Cyanobacterial Fluorescence

Nonphotochemical quenching reflects any process other than photochemistry which lowers the yield of variable fluorescence. It can be quantified by using the coefficient q_N (145):

$$q_N = 1 - [(F_M' - F_O')/(F_M - F_O)] = 1 - (F_V'/F_V)$$

q_N compares the span of variable fluorescence under a given condition, $F_V' = (F_M - F_O')$, with the maximum potential variable fluorescence, $F_V = (F_M - F_O)$ (Fig. 2). In cyanobacteria, variable fluorescence appears to arise essentially from PS II, while as discussed, the F_O' and F_O fluorescence levels arise only partly from PS II. The cyanobacterial F_O' and F_O signals detected by a modulated fluorometer with a red-modulated light-emitting diode (LED) each contain a contribution from phycocyanin fluorescence, which we believe is fairly constant over the course of a measurement. This underlying background fluorescence is subtracted out as an equivalent component of both F_O' and F_O and so does not seriously distort the calculation of q_N.

Significant drops in F_O fluorescence during illumination can distort q_N in higher plants. This F_O quenching is quantified as follows (9):

$$1 - q_O = F_O'/F_O$$

A modified calculation of the q_N coefficient compensates for q_O (9):

$$q_N = 1 - \{F_V'/[F_V (F_O'/F_O)]\}$$

In cyanobacteria, F_O' is usually close to or somewhat higher than F_O ('negative' q_O), and both q_N expressions give similar results.

An alternate quantification of nonphotochemical fluorescence quenching is as follows (67):

$$NPQ = (F_M - F_M')/F_M'$$

This is a Stern-Volmer formulation that measures the ratio of quenched to remaining fluorescence. The absolute value of NPQ does suffer from distortion by the underlying phycobiliprotein fluorescence, which contributes to both F_M and F_M'. This potential problem may be addressed by fluorescence measurements with alternate modulated light sources, such as a blue LED (65), which allow excitation of chlorophyll fluorescence without interference from phycobiliprotein emissions. The use of NPQ also avoids the problematic measurement of F_O' and is therefore possibly a preferable measure of nonphotochemical quenching in cyanobacteria. Plots of q_N and NPQ derived from the same fluorescence traces generally show similar although not identical patterns.

Figure 7 presents typical light response curves of nonphotochemical quenching in the cyanobacterium *Synechococcus* sp. strain PCC 7942, along with a curve from a rye plant for comparison. In the cyanobacterium, q_N (or NPQ) is high in the dark and drops to a minimum near the growth light intensity. In the plant, q_N climbs steadily as the light intensity surpasses the growth level. These differing patterns reflect a fundamental difference in the predominant processes contributing to nonphotochemical quenching. In plants nonphotochemical quenching is dominated by a mechanism(s) for excitation-dependent thermal dissipation of energy from PS II and its antennae, in competition with fluorescence and photochemistry (1, 46, 47, 57, 66, 67, 153).

In contrast, nonphotochemical quenching in cyanobacteria

largely reflects changes in the PS II fluorescence yield as a result of the state transition mechanism (7, 14, 17, 18, 21, 22, 33, 97, 111, 112), which regulates the distribution of excitation energy between PS II and PS I. The biophysical basis of the energy redistributions remains incompletely understood (2, 17, 18, 118, 156), but it is clear that the relative distribution of excitation energy from the phycobilisome to the two photosystems changes (7, 42, 136). An allophycocyanin-B protein in the phycobilisome core serves as a regulated secondary terminal emitter, which receives about 25% of the excitation energy captured by the phycobilisome. Under state I, this portion of the captured excitation is directed largely to PS II, but under state II, most of it is redirected to PS I, thereby lowering the yield of PS II fluorescence and photochemistry (48, 49, 112, 156). A recent model proposes that reversible changes in the oligomerization of PS II and PS I underpins the state transition mechanism (7). Furthermore, the phycobilisomes diffuse along the surface of the thylakoids (96) sufficiently rapidly that movement of phycobilisomes could be involved in the state transitions.

State transitions in cyanobacteria are regulated by the redox status of the electron transport chain joining PS II and PS I (34, 41, 94, 95, 97, 128, 149). If the chain is reduced, cells tend to state II, with a low yield of PS II fluorescence and a distribution of excitation energy to PS I, which extracts electrons from the chain. If the chain becomes more oxidized the cells shift toward state I, with a higher yield of PS II fluorescence and more distribution of excitation energy to PS II. Although the evidence for redox control of state transitions is quite strong, it is possible to observe conditions of low q_P, indicating PS II reduction, while cells maintain low q_N, presumed to reflect state I and an oxidized intersystem transport chain (86a). To accommodate these observations within the framework of redox regulation of state transitions, there must be partial decoupling of PS II and the intersystem redox status under some conditions, or some as yet unknown subtlety to the redox sensing mechanism(s).

Respiratory and photosynthetic electron flow occur via the same electron transport intermediates in cyanobacteria (5, 44, 55, 85, 86, 131, 135, 147) (Fig. 1). Respiratory electron flow in the dark generally poises the electron transport chain toward a reduced state; therefore, in the dark or under very low light, cyanobacteria are in state II (41, 94, 95). This is reflected in low variable fluorescence and high nonphotochemical quenching, as shown in Fig. 7. As light is applied, PS I activity partially oxidizes the electron transport chain and the cells shift toward state I, with higher PS II fluorescence yield (Fig. 2) and lower nonphotochemical quenching (Fig. 7). As light exceeds the growth level, the PS II variable fluorescence yield remains high or drops somewhat (Fig. 2) and nonphotochemical quenching may increase, although not to the levels achieved in darkness (Fig. 7). Finally, if DCMU is added (Fig. 2), PS II centers close, resulting in a rapid rise in fluorescence as photochemical quenching is lost. This rapid rise is equivalent to the fluorescence peak under a brief saturating light pulse, which also closes reaction centers (Fig. 2). The DCMU inhibition of PS II substantially lowers electron input to the transport chain, and the electron transport chain becomes oxidized by continuing PS I activity. This results in a second, slower fluorescence rise phase (Fig. 2) as the cells enter a full state I with maximum PS II fluorescence (F_M) (Fig. 2) and minimal nonphotochemical quenching. This redox dependence of the state transition is illustrated in Fig. 8. Titration with the inhibitor DCMU (143) under the growth light intensity progressively blocks PS II activity (Fig. 6), resulting in net oxidation of the transport chain and state I with low q_N (Fig. 8A). Titration with DBMIB (143) also inhibits electron transport, but PS II itself remains active, resulting in reduction of the portion of the transport chain preceding the DBMIB binding site on the cytochrome *bf* complex. The state transition mechanism senses this change as an apparent overexcitation of PS II, and the cells enter state II with high q_N (Fig. 8B).

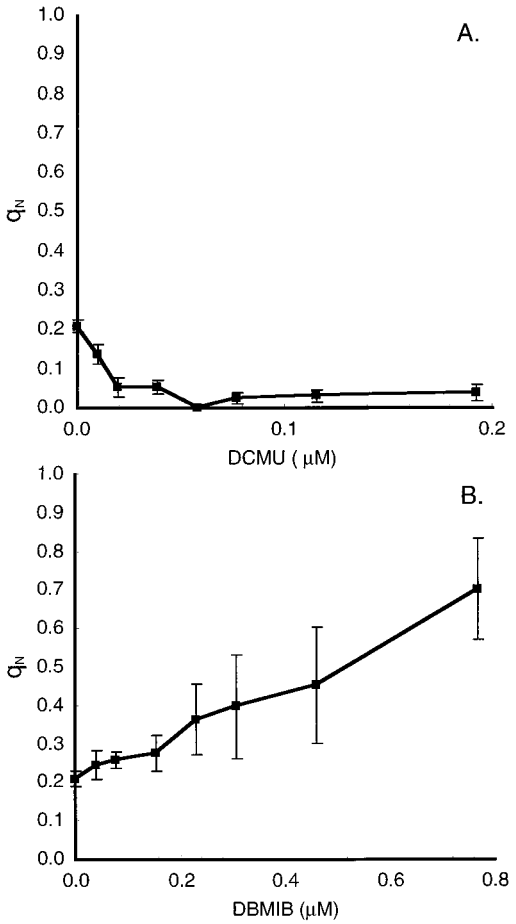

FIG. 8. Nonphotochemical quenching of fluorescence under DCMU (A) and DBMIB (B) treatments. Modulated fluorescence traces (Fig. 2) were measured under the growth light intensity of 50 μmol of photons m^{-2} s^{-1}. Results are means and standard errors of measurements on the same culture at 37°C (n = 3). Modified from reference 22 with permission of the publisher.

The origin of cyanobacterial nonphotochemical quenching in the state transition mechanism is illustrated in Fig. 9, which compares the 77K fluorescence emission spectra and q_N values of a cyanobacterial sample in the darkness, under growth light intensity, and after addition of DCMU. The 77K emission spectra, measured with excitation of the phycobilisome at 574 nm, show the changing distribution of energy. In the dark, PS II fluorescence is low, reflecting the high nonphotochemical quenching. Upon illumination, q_N drops as the excitation energy is redistributed in favour of PS II. This process continues to an extreme upon addition of DCMU.

In plants, excitation-dependent quenching driven by the *trans*-thylakoid ΔpH gradient is the major component of q_N (1, 46, 47, 57), with only minor contributions from state transitions and other mechanisms (66). In plants, nonphotochemical quenching drops upon collapse of the *trans*-thylakoid pH gradient (67, 153). In cyanobacteria, nonphotochemical quenching does not collapse upon addition of carbonyl cyanide

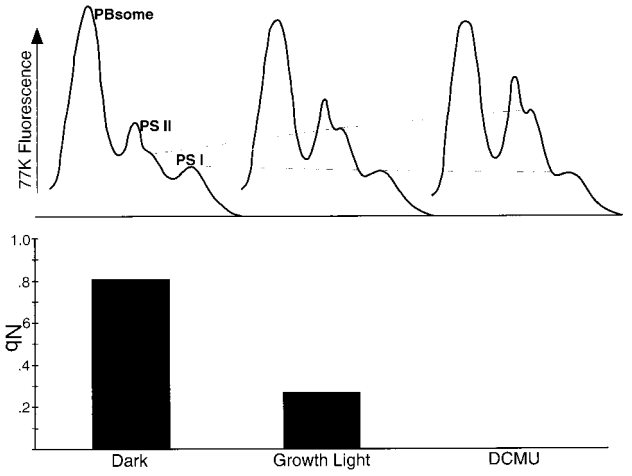

FIG. 9. 77K Fluorescence emission spectra show that changing q_N reflects state transitions. The dashed lines emphasize increasing PS II fluorescence and decreasing PS I fluorescence in parallel with the drop in nonphotochemical quenching, measured in the same cultures. A *Synechococcus* sp. strain PCC 7942 culture sample was incubated in the PAM cuvette in darkness, under the growth light intensity (50 μmol of photons m^{-2} s^{-1}), and after addition of DCMU under continuing illumination. For each condition, q_N was measured and a small sample was taken for 77K fluorescence emission spectral analysis. Excitation of 77K fluorescence was carried out with 574 nm light absorbed by the phycobilisome (PBsome). Fluorescence spectra are from samples of equal chlorophyll content but are not otherwise normalized.

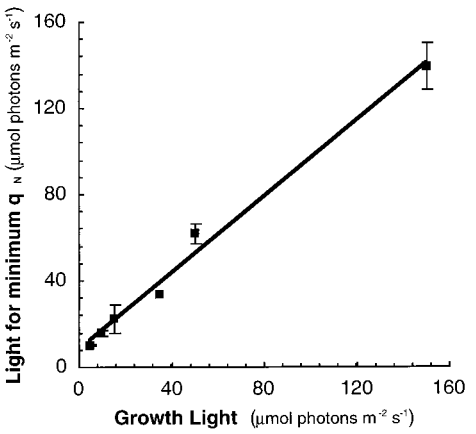

FIG. 10. Near the growth light intensity, q_N reaches a minimum for a wide range of cyanobacterial strains and culture conditions. Mean values are plotted for strains grown at 5 (n = 3), 10 (n = 5), 15 (n = 6), 35 (n = 1), 50 (n = 20) and 150 (n = 3) μmol of photons m^{-2} s^{-1}. The strains were *Anabaena*/*Nostoc* sp. strain 7120, *Calothrix* sp. strain PCC 7601, *Nostoc* sp., *Pseudanabaena* sp. strain PCC 6901, *Synechococcus* sp. strain PCC 7942, *Synechococcus* sp. strain PCC 6301, and *Synechocystis* sp. strain PCC 6701. Modified from reference 22 with permission of the publisher.

p-trifluoromethoxyphenylhydrazone (FCCP), an ionophore uncoupler (22, 86a). We therefore concluded that energy-dependent quenching is not a significant contributor to q_N in cyanobacteria. Interestingly, Delphin et al. (32) present evidence that in red algae, whose chloroplasts also contain phycobilisomes, a plant-type ΔpH-dependent nonphotochemical quenching occurs even under low light levels, in contrast to the state transitions observed in cyanobacteria, which do not depend on ΔpH.

Nonphotochemical quenching reflects changes in PS II photochemistry but not necessarily net energy dissipation from the photosynthetic system, if energy is redirected from PS II to PS I. In cyanobacteria, therefore, a high q_N does not necessarily mean a low overall photosynthetic efficiency (21, 138). Indeed, redistribution of energy from the PS II-phycobilisome supracomplex to PS I is an important regulatory mechanism in cyanobacteria, to accommodate changing excitation (21, 132, 133) or requirements for ATP to accumulate CO_2 and nutrients (5, 91, 116). In contrast to this work, nonphotochemical quenching in a *Microcystis* strain has been interpreted as reflecting thermal energy dissipation of excitation. These cyanobacteria had a sustained content of the carotenoid zeaxanthin (60). Furthermore, some cyanobacterial strains such as *Synechocystis* sp. strain PCC 6803 do not display high nonphotochemical quenching after dark adaptation, indicating that they do not enter state II in the dark (51).

The diversity of cyanobacterial pigment and photosynthetic systems requires further characterization of the various origins and regulation of nonphotochemical quenching, particularly under natural growth conditions and limiting nutrient conditions, which can also lead to the loss or alteration of the state transition response (29, 39).

Recent evidence suggests that state II in dark-adapted cyanobacteria might involve an inactivation of the water-splitting complex (83). This limits the electron supply to the P_{680} chl of PSII and favors recombination in the reaction center, lowering the fluorescence yield (112a). This interpretation suggests that state transitions involve several different mechanisms, since there is clear evidence that under some conditions redirection of excitation energy is responsible for the changes in fluorescence yield observed as state transitions (see, e.g., reference 21).

A methodological problem in measuring light response curves of q_N is the potential photoinhibitory loss of variable fluorescence as the actinic light is increased above the acclimated growth level. This can be detected if the F_M level measured with DCMU is lower than a previous F_M' level, usually that measured around the growth light intensity. In this case, the uninhibited F_M level for the sample is unknown, and therefore absolute values of q_N cannot be determined. Nevertheless, if a relative q_N is calculated by using the highest F_M' level achieved, the pattern with respect to light intensity is valid. Photoinhibition during measurement can be avoided by minimizing saturating flashes, keeping exposure to high actinic light brief, and terminating light response curves at 5 to 10 times the growth light intensity.

Predicting the Acclimated Light Intensity from q_N Light Response

Figure 10 shows that in a wide range of cyanobacteria with different pigment contents and morphologies, grown under different conditions and light histories, q_N reaches a minimum near the acclimated growth light intensity (22). Therefore, the light intensity to which the population is photosynthetically acclimated can be predicted from a readily measured light response curve of q_N (or NPQ). Although ambient light is readily measured under many circumstances, this relation between q_N and growth light shows the range of the overall light regime which is exploited for acclimated growth. In many cases, the natural light regime is highly variable and cyanobacteria must integrate light information over time to regulate synthesis of the abundant proteins of the photosynthetic system. We hope that the q_N light response curve will prove useful with samples where the past light regime is unknown or samples from variable light regimes in which the optimal light

intensities for acclimated growth are unknown (132, 133). For controlled light acclimation studies, tracking the q_N minimum over time may show the point at which a population completes acclimation after a light shift. Furthermore, for prediction of electron transport in cyanobacteria from fluorescence parameters, it is essential to measure fluorescence under approximately the acclimated growth light intensity (see below).

Note that this relation does not involve the absolute levels of q_N or NPQ but simply their pattern in response to light intensity. Determination of actual q_N or NPQ levels requires the measurement of F_M by destructive DCMU treatment. If a nondestructive measurement is required and the absolute levels of the parameters are not critical, a simple plot of F_M' or F_V'/F_M' against light intensity will suffice; the light intensity at which maximum values are achieved coincides approximately with the acclimated light intensity.

Inorganic Carbon Accumulation and Fluorescence Quenching

Cyanobacteria and some algae accumulate an intracellular pool of inorganic carbon to limit photorespiration (4, 109). The size of the pool influences both photochemical and nonphotochemical fluorescence quenching (5, 87–91). These fluorescence effects can be used to monitor the transport and accumulation of inorganic carbon noninvasively and in real time (30, 90). One mechanism for the fluorescence effects appears to be a bicarbonate-dependent stimulation of linear electron flow from PS I to O_2, CO_2, or nitrite in some strains, which increases photochemical quenching (5, 51, 77, 78, 88, 91, 92).

Carbon accumulation can also influence nonphotochemical quenching, possibly by driving a transition to state II to increase PS I cyclic electron transport to fulfill the need for ATP (93) to drive the accumulation pump.

Miller et al. (89) also describe a form of nonphotochemical quenching which depends on inorganic carbon accumulation in *Synechococcus* sp. strain PCC 7942 cells grown at high light intensities but which does not occur in cells grown at low light intensities. The modulated fluorescence trace from the cells grown at high light intensities resembles a transition to state II upon carbon accumulation, with a drop in F_M' fluorescence yield and an increase in q_N. This increase in q_N was not, however, reflected in a comparable change in the cellular fluorescence emission spectra at 77K. This is in contrast to a large drop in 77K PS II fluorescence emission upon dark adaptation of the same cells, similar to that presented in Fig. 9. This component of carbon accumulation-dependent nonphotochemical quenching in cells grown at high light intensities thus appears distinct from the state transition mechanism. It might ultimately relate to bicarbonate-dependent changes in the PS II water splitting-complex (112a).

State Transitions Can Be Measured Nondestructively

The change in fluorescence yield during state transitions is strongly influenced by the excitation light, the rate of respiration, the cellular iron supply (39), and the circadian status of the cell (83). Therefore, determining the size of state transitions can provide information about these factors. The magnitude of state transitions can be measured by comparing q_N or NPQ values under different conditions, but explicit calculation of these parameters requires a lethal DCMU treatment to determine F_M. This precludes repeated measures on a single sample over time. Alternately, changes in q_N between different conditions, for example cells in the dark and under illumination, can be quantified by a nondestructive method that does not require explicit measurement of F_M:

$$(1 - q_{N_{light}})/(1 - q_{N_{dark}}) = (F_M' - F_O')/(F_{Mdark} - F_O)$$

In general, for the strains we have studied, this ratio is maximal for a given sample if measured by using the growth light intensity. During prolonged dark incubation, this ratio declines toward 1, as the F_{Mdark} level approaches but does not reach F_M'. Thus, during dark incubation, the state transition gradually disappears. This probably reflects the progressive consumption of reserves used to support electron transport in the dark (34, 94, 123). The presence or size of state transitions can also be influenced by iron or nitrogen stress (29, 39), opening the possibility of using the state transition as a noninvasive monitor of these aspects of physiology.

PREDICTING PHOTOSYNTHESIS FROM FLUORESCENCE

For field measurements, a fluorescence-based estimate of electron transport and carbon dioxide fixation is very valuable (104, 134). Fluorescence measurements are possible with dilute samples; unlike gas exchange, they are specific to photobionts and so do not detect interference from heterotrophic respiratory activity in mixed samples or lichens. The fluorescence transients arise largely from PS II, and so calculations based on fluorescence reflect PS II activity and electron transport through PS II. In extrapolating from fluorescence signals to photosynthesis, we therefore rely on a congruence between PS II activity, net electron transport, and overall photosynthesis. Cyanobacteria and cyanolichens have carbon-concentrating mechanisms which suppress the oxygenase reaction of the ribulose-1,5-bisphospate oxygenase-carboxylase enzyme (4, 5, 87, 90, 91, 108, 110). This simplifies the empirical relation between PS II activity, reflected in fluorescence signals, and gas exchange, as is also the case in C_4 plants (36).

Sundberg et al. (138) have simultaneously measured fluorescence quenching parameters and CO_2 exchange in cyano-lichens to develop a model to predict gross photosynthesis from fluorescence parameters. They found the empirical relation

$$P = \phi\text{PS II} \times I_i \times 1\ CO_2\ \text{fixed}/10\ \text{photons}$$

where P = micromoles of CO_2 fixed per square meter per hour, ϕPS II = $(F_M' - F_S)/F_M' = (F_V'/F_M')q_P$ (a fluorescence estimate of the photochemical yield of PS II), I_i = number of incident photons per square meter per hour, and 1 CO_2 fixed/10 photons is an empirical conversion factor.

This predictor gave good estimates of actual CO_2 fixation near the acclimated growth light intensity. Under higher light, the predictor, which is based on light-driven electron flow through PS II, progressively overestimated actual CO_2 fixation. The overestimation probably reflects electron flow back to O_2 under excess light (22a, 88, 131, 147), which maintains PS II centers open but does not contribute to CO_2 CO_2 fixation. Other workers have found that this flexible electron transport to O_2 interferes with estimates of CO_2 fixation from fluorescence measures (74). In our experiments, we could estimate the acclimated growth light intensity from the light response curve of nonphotochemical quenching. With this light intensity, we made reasonable predictions of the acclimated rate of CO_2 fixation from fluorescence parameters. The general applicability of this approach must, however, be further tested. The 1 CO_2 fixed/10 photons is an empirical conversion factor that reflects the (unknown) quantum yield of CO_2 fixation and also compensates for the downward distortion of F_V'/F_M' by phycobilisome fluorescence.

We developed a similar empirical relation to predict gross oxygen evolution from ϕPS II in liquid cyanobacterial cultures:

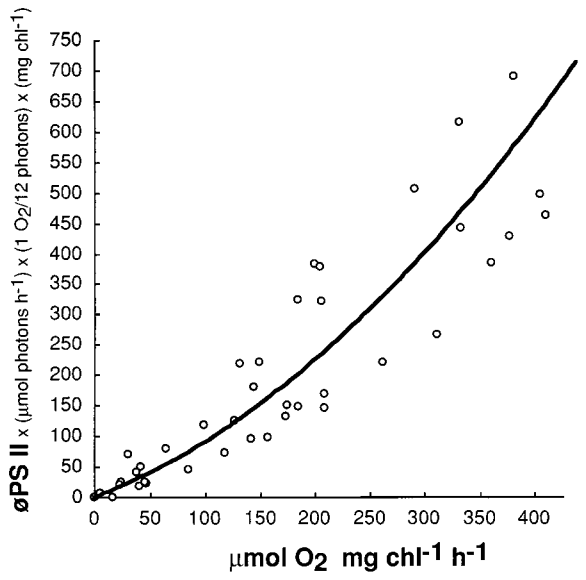

FIG. 11. ϕPS II reflects O_2 evolution. *Synechococcus* sp. strain PCC 7942 was grown under 50 μmol of photons m^{-2} s^{-1} at 37°C and then incubated under a range of light intensities. Gross oxygen evolution (micromoles of O_2 per milligram of chlorophyll per hour) was estimated as light-dependent oxygen evolution minus dark uptake. ϕPS II = $(F_M' - F_S)/F_M' = (F_V'/F_M')q_P$. I_i = micromoles of photons incident on the sample per hour.

$$\text{Oxygen evolution} = \phi PS\ II \times I_i \times 1\ O_2/12\ \text{photons} \times 1/\text{chl}$$

where oxygen evolution is expressed as micromoles of O_2 per milligram of chlorophyll per hour, ϕPS II = $(F_M' - F_S)/F_M' = (F_V'/F_M')q_P$, I_i = number of micromoles of photons incident per hour; 1 O_2/12 photons is an empirical conversion factor, and chl is the chlorophyll content in milligrams.

Figure 11 shows that this relation gives a good approximation of measured oxygen evolution at or near the growth light intensity. At higher light intensities, the predictor increasingly overestimates measured oxygen evolution, again because of pseudocyclic electron flow, with electrons extracted from water by PS II ultimately reaching oxygen as a terminal acceptor. The empirical conversion factor of 1 O_2/12 photons again combines the unknown quantum yield of O_2 evolution and compensation for the low F_V'/F_M' values in cyanobacteria.

In summary, a two-step process gives reasonable estimates of oxygen evolution or carbon fixation under the acclimated growth light intensity. First, a light response curve of nonphotochemical quenching shows the acclimated light intensity. Then oxygen evolution or carbon fixation is estimated from the fluorescence parameter ϕPS II, the acclimated light level, and an empirical conversion factor calibrated against gas exchange measurements. Failure to measure near the growth light intensity, or an inappropriate empirical calibration factor, can lead to a significant overestimation of actual photosynthesis.

APPLYING FLUORESCENCE ANALYSIS TO DIFFERENT CYANOBACTERIA

We have tested the methods described in nine strains of cyanobacteria representing a wide range of pigment contents, phycobilisome structures, and physiological properties, as well as six strains of cyanobacterial lichens, as outlined in Table 2. The methods have been extensively validated with *Synechococcus* sp. strain PCC 7942 (*Anacystis nidulans* R2) grown under a range of light intensities, CO_2 levels, and temperatures (22–24, 26–28; see above). Furthermore, a mutant of this strain lacking phycobilisome rods and containing no PC proved amenable to quenching analysis (Fig. 3) (158). The closely related strain *Synechococcus* sp. strain PCC 6301 (*Anacystis nidulans*) dis-

TABLE 2. Cyanobacteria and cyanolichens used for the modulated fluorescence analyses described in this review

Species	Growth light	Phycobiliprotein[a]
Cyanobacteria		
Anabaena/Nostoc sp. strain PCC 7120	Fluorescent (50 μmol of photons m^{-2} s^{-1})	APC, PC, PEC
Calothrix/Tolypothrix/Fremyella sp. strain PCC 7601	Fluorescent (10 μmol of photons m^{-2} s^{-1})	APC, PC, PE
Calothrix/Tolypothrix/Fremyella sp. strain PCC 7601	Green (15 μmol of photons m^{-2} s^{-1})	APC, PC, PE
Calothrix/Tolypothrix/Fremyella sp. strain PCC 7601	Red (15 and 35 μmol of photons m^{-2} s^{-1})	APC, PC
Gloeobacter violaceus PCC 7421	Fluorescent (10 μmol of photons m^{-2} s^{-1})	APC, PC, PE, PUB
Pseudanabaena sp. strain PCC 6901	Fluorescent (50 μmol of photons m^{-2} s^{-1})	APC, PC
Synechococcus sp. strain PCC 7942 (*Anacystis nidulans* R2)	Incandescent (10, 15, 50, and 150 μmol of photons m^{-2} s^{-1})	APC, PC
Synechococcus sp. strain PCC 7942 phycobilisome-minus	Incandescent (50 μmol of photons m^{-2} s^{-1})	APC
Synechococcus sp. strain PCC 6301 (*Anacystis nidulans*)	Fluorescent (10 and 50 μmol of photons m^{-2} s^{-1})	APC, PC
Synechocystis sp. strain PCC 6701	Fluorescent (50 μmol of photons m^{-2} s^{-1})	APC, PC, PE
Nostoc from lichen *Peltigera canina*	Fluorescent (5 μmol of photons m^{-2} s^{-1})	APC, PC, (PE, PEC?)
Cyanolichens (*Nostoc*)		
Leptogium coralloideum	Natural	APC, PC, (PE, PEC?)
Lobaria scrobiculata	Natural	APC, PC, (PE, PEC?)
Nephroma bellum	Natural	APC, PC, (PE, PEC?)
Peltigera malacea	Natural	APC, PC, (PE, PEC?)
Peltigera neopolydactyla	Natural	APC, PC, (PE, PEC?)
Nephroma arcticum (*Nostoc* and green algae *Coccomyxa*)	Natural	APC, PC, (PE, PEC?)

[a] APC, allophycocyanin; PC, phycocyanin; PE, phycoerythrin; PEC, phycoerythrocyanin; PUB, phycoerythrin containing phycourobilin chromophores.

plays very similar fluorescence properties (21a). Both these strains have a somewhat unusual phycobilisome with a core composed of only two cylinders.

Calothrix sp. strain PCC 7601 is a heterocystous, filamentous strain which strongly regulates its content of phycoerythrin and phycocyanin according to the relative supply of green and red light. Quenching analysis has shown that when this strain is grown under green light, energy captured by phycoerythrin is transferred from the phycobilisome to PS I to maintain balanced electron transport. This transfer is reflected in a high q_N and serves as a good demonstration that in a cyanobacterium a high q_N does not usually reflect excitation dissipation but, rather, reflects the transfer of excitation to PS I at the expense of PS II. Upon transfer to red light or prolonged growth under red light, this excitation transfer stops, the PS II fluorescence yield increases, and q_N drops (21).

Four other diverse cyanobacterial strains have also shown responses similar to that of *Synechococcus* sp. strain PCC 7942 (21a): *Anabaena*/*Nostoc* sp. strain PCC 7120 is a heterocystous, filamentous strain with another atypical phycobilisome structure (35), *Nostoc* sp. is a strain originally isolated from the lichen *Peltigera canina*, *Synechocystis* sp. strain PCC 6701 is a unicellular strain which contains phycoerythrin; and *Pseudanabaena* sp. strain PCC 6901 is a gas-vacuolated strain forming short filaments.

Six lichens with *Nostoc* strains as symbionts have also proved amenable to quenching analysis by using a Hansatech cuvette designed for leaf discs; *Leptogium coralloideum*, *Lobaria scrobiculata*, *Nephroma bellum*, *Peltigera malacea*, *Peltigera neopolydactyla*, and the cephalodia regions of *Nephroma arcticum*, a tri-partite lichen with the green algae *Coccomyxa* and *Nostoc*. In lichens, the dark-light state II-state I transition tends to be small, probably reflecting fungus-cyanobacterium interactions in respiration and carbohydrate consumption (138).

Goosney and Miller (51) found that the widely studied facultative heterotrophic strain *Synechocystis* sp. strain PCC 6803 shows little or no increase in F_M' upon illumination under most conditions. The fluorescence induction trace in this strain is rather plant-like in that F_M' measured in dark-adapted cells is close to F_M. This distinct pattern might reflect differences in this strain in the redox balance of intersystem electron transport in the dark, such that the cells do not enter state II in the dark. This plant-like induction pattern upon illumination can occur in other strains, particularly when dark respiration is slow as under conditions of low carbohydrate reserves or under nutrient stress (29, 39). Alternatively, there may be more fundamental distinctions in the organization of light capture and electron transport between various strains.

Interestingly, the unusual cyanobacteria *Gloeobacter violaceus* PCC 7421 was the only strain surveyed for which quenching analysis proved impossible. This strain lacks thylakoids (115) and instead of typical phycobilisomes contains simpler rod-like phycobiliprotein structures (52) associated with the plasma membrane. In our measurements, this strain showed almost no variable fluorescence even when growth and oxygen evolution were readily measurable (21a). 77K fluorescence spectra from this strain also lack the expected long-wavelength PS I emission, even though the presence of PS I was verified functionally (64). Clearly, the unusual photosynthetic system of this cyanobacterium results in distinct fluorescence properties.

CONCLUSIONS AND PROSPECTS

Tracking Acclimation Status in the Laboratory and Field

Fluorescence analysis is an integral part of studies of photosynthesis in cyanobacteria and other organisms. In recent years, advances in instrumentation and interpretation have greatly expanded the applications of fluorescence to ecophysiological and molecular studies (6, 13, 40, 99).

For cyanobacteria, estimating the acclimated light in a population from the light response of nonphotochemical quenching (22) will allow rapid tracking of acclimation in laboratory experiments or field studies. Cells must integrate changing environmental signals (132, 133) to regulate the expression of abundant proteins such as ribulose-1,5-bisphosphate carboxylase/oxygenase or phycobilisomes, in order to produce appropriate long-term levels of protein. The use of fluorescence to monitor acclimation (10, 61, 73, 74, 130) may show how cyanobacteria and cyanolichens set their targets for gene expression and metabolic acclimation in the face of changing light, environmental factors, and circadian status.

The size of the dark-to-light state transition is strongly influenced by cellular respiration, which poises the electron transport chain (34, 55, 84–86, 94, 95, 131, 135). Therefore, the state transition may provide estimates of respiration and indirectly of the reserves available to support respiration. Further work in this area might lead to rapid measures of the levels of reserves in cyanobacteria or cyanolichens, as they fluctuate diurnally or seasonally (123, 124). Estimates of reserves are valuable in understanding physiological and differentiation responses of cyanobacteria and also in determining production available for export from the community.

The size of the dark-to-light state transition is also strongly influenced by cellular iron and nitrogen status (29, 39). Under iron limitation, which is widespread in nature (8), cyanobacteria produce alternate chlorophyll-protein complexes associated with PS II (70, 75, 76), which leads to suppression of the state transition (39). Therefore, the size of state transition may be useful as a measure of iron limitation.

The iron example illustrates that as we apply fluorescence analysis to natural cyanobacteria, nutrient or reserve limitations may greatly alter fluorescence signals from those observed with laboratory cultures. Thus, further work is needed on measuring and interpreting fluorescence signals under nonoptimal and natural conditions, where pigment composition and cellular organization may be very different from typical laboratory cultures. Simultaneous parallel detection of fluorescence transients at several excitation and emission wavelengths (61, 65) holds great promise for the study of mixed populations or strains with complex pigment compositions.

Conclusions

Chlorophyll fluorescence signals from cyanobacteria and cyanolichens show patterns very distinct from those of green plants. Therefore, the fluorescence measurements and analyses originally developed for green plants must be modified, but cyanobacterial fluorescence also yields information not accessible from plant fluorescence signals. The cellular phycobiliprotein content influences the F_O level fluorescence, particularly when phycobiliprotein levels are high. This leads in some cases to downward distortion of the parameter F_V/F_M, which is widely used as an index of PS II activity. The photochemical quenching coefficient, q_P, provides a robust index of the balance between excitation of PS II and electron transport and shows that cyanobacteria have a high and flexible capacity to remove electrons from PS II. Nonphotochemical quenching of

PS II variable fluorescence in cyanobacteria reflects the state transition mechanism for distribution of excitation between the photosystems. A characteristic decline of nonphotochemical quenching during a shift from dark to increasing light provides a valuable means of estimating the light level to which a cyanobacterial population is photosynthetically acclimated. Gross oxygen evolution or carbon fixation can be estimated in at least some cases (138; but see reference 74)) from the fluorescence parameter ϕPS II, the acclimated growth light level, and an empirically verified apparent quantum yield.

APPENDIX

Cyanobacterial Cultures and Pigment Measurements

Cyanobacteria were grown in BG-11 inorganic medium (114), supplementally buffered with 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) to a final pH of 7.5. *Synechococcus* sp. strain PCC 7942 cultures (300 ml) were grown in flat flasks bubbled with 5% CO_2 in air (about 1 ml s^{-1}) at 37 or 25°C with continuous, even illumination of 10, 15, 50, or 150 μmol of photons m^{-2} s^{-1}. For *Synechococcus* sp. strain PCC 7942, the chlorophyll and phycocyanin contents were determined by using whole-cell spectra as described by Myers et al. (98), corrected for scattering by subtracting the absorbance at 750 nm from the chlorophyll and phycocyanin peaks. Whole-cell spectra are less useful for filamentous strains, since significant and variable light scattering distorts spectra. For these strains, chlorophyll was extracted in methanol and measured by the method of Tandeau de Marsac and Houmard (140). For the other strains used in the q_N measurements shown in Fig. 9, the growth conditions were generally similar but the temperature ranged from 18 to 37°C, the light intensity ranged from 5 to 150 μmol of photons m^{-2} s^{-1}, either fluorescent or incandescent, and the CO_2 supply ranged from ambient to 5% in air.

Modulated Fluorometer Configuration and Measurement Procedure

In our experiments, chlorophyll *a* fluorescence induction was measured with a pulse amplitude modulated fluorometer (128, 129) (PAM chlorophyll fluorometer; Walz, Effeltrich, Germany) with the PAM 103 accessory and a Schott KL1500 lamp (Schott, Mainz, Germany) to provide saturating flashes. The recording device, in our case a chart recorder, should respond to changes within less than 100 ms (128). A PAM-compatible system of cuvette, magnetic stirrer, oxygen electrode, and halogen incandescent actinic lamp were used for the simultaneous measurement of fluorescence and oxygen evolution (Hansatech, King's Lynn, United Kingdom) (151). Simultaneous measurement strengthens the conclusions which can be drawn, since the two techniques are complementary. This cuvette system allows measurement of liquid samples down to concentrations of about 0.2 μg of chlorophyll/ml, suitable for laboratory cultures or somewhat concentrated natural samples. An alternate cuvette (Walz ED-101 US) (125) uses a smaller vessel and light guides to extend the functional concentration range to less than 1 μg of chlorophyll per liter, allowing direct measurement of many natural water samples. The actinic beam and fluorescence detector are set at 90° in this system, rather than at 180° as in the Hansatech cuvette. This alternate geometry may result in changes in some measurements (60a), and so the influences of cuvette geometry on signals are an issue for further research. A further innovation is a system involving light-emitting diodes of different wavelengths, for preferential excitation of specific pigments (65). This technique shows promise for resolution of fluorescence signals from different photobionts in mixed samples. Comparable modulated fluorometers, with variations in the nature and wavelength of the measuring and actinic lights and in the cuvettes, are available from several companies.

Culture samples, generally at around 2 μg of chlorophyll per ml, were dark adapted for 5 min in the Hansatech cuvette. The analysis procedure is outlined in Fig. 2. Minimum fluorescence, F_O, was determined by illuminating the dark-adapted cells with a low-intensity light modulated at 1.6 kHz (average intensity, 0.14 μmol of photons m^{-2} s^{-1}) from a light-emitting diode (peak emission, 655 nm). Fluorescence was detected at wavelengths greater than 700 nm. The intensity of the measuring beam should be checked to ensure that it is sufficiently weak to avoid electron transport, detected as changes in the oxygen electrode response (20). A 1-s flash of saturating white light (8,000 μmol of photons m^{-2} s^{-1}) was then given to determine the maximal fluorescence in the dark-adapted state, $F_{M\mathrm{dark}}$, with all PS II centers closed by the saturating flash. In cyanobacteria, $F_{M\mathrm{dark}}$ is generally significantly lower than the maximal fluorescence, F_M (20, 22, 23, 27, 28, 91), although this is not the case in all strains under all conditions (see, e.g., reference 51). After a further 30 s, the actinic light was activated. Steady-state fluorescence, F_S, was reached within 2 min. Minimum fluorescence in the light-adapted state, F_O', was then measured by briefly interrupting the actinic beam and either leaving culture in darkness for ca. 5 s or applying weak far-red light (ca. 5 μmol of photons m^{-2} s^{-1}; >700 nm) to excite PS I activity and extract electrons from the transport chain. Both procedures for measuring F_O' gave similar results in cyanobacterial measurements. The actinic light was then resumed, and after F_S was reestablished, a saturating light pulse was given to again close all PS II centers, driving photochemical quenching to zero for determination of maximal fluorescence in the light-adapted state, F_M'. The actinic light intensity was then increased, and the process was repeated sequentially to generate a light response curve.

Cyanobacterial cells have CO_2-concentrating mechanisms (CCM) (4, 87), and when this system is induced the cells do not usually become CO_2 limited during short measurements. Laboratory cyanobacterial cultures are often supplemented with high CO_2 levels, and under these growth conditions the CCM activity is repressed. In these cases, photosynthesis may deplete the dissolved inorganic carbon supply of the cuvette volume, resulting in CO_2 limitation of photosynthesis. Even in cells grown under low inorganic carbon, prolonged photosynthesis in a closed cuvette can deplete the inorganic carbon sufficiently to cause carbon limitation. Furthermore, electron flow to oxygen is often dependent on the presence of an intracellular biocarbonate pool, so CO_2 limitation can also limit electron flow to oxygen as a terminal acceptor (88). The onset of CO_2 limitation results in steady-state F_S fluorescence increasing toward F_M', in parallel with a decline in the oxygen evolution rate. Cultures grown under CO_2 supplementation were therefore routinely supplemented with 7 mM NaH_2CO_3 at the start of the measurement.

Finally, DCMU (final concentration, 0.5 μM) was injected into the cuvette to bind to PS II centers (143). This leads to a rapid rise in fluorescence to a level similar to F_M', as PS II closes, photochemistry is blocked, and photochemical quenching is lost (Fig. 2). The loss of PS II activity leads to oxidation of the plastoquinone pool, which in turn drives a slower fluorescence rise phase (Fig. 2) as the cells go to full state I with maximal fluorescence, F_M. The concentration of DCMU required for rapid PS II closure is somewhat dependent on the

strain and growth conditions and should be verified empirically. The parameters F_O, $F_{M\text{dark}}'$ F_S, F_O', F_M', and F_M were used for the calculation of photochemical (q_P) and nonphotochemical (q_N) quenching (145), and the apparent efficiency of excitation energy capture by open PS II reaction centers (F_V'/F_M') (45).

Figure 2 is a fluorescence trace from our usual protocol for measuring room temperature fluorescence in *Synechococcus* sp. strain PCC 7942, showing a typical cyanobacterial response over a series of increasing light intensities. The number of saturating flashes is kept to a minimum to avoid photoinhibition or sustained induction of electron transport. Schreiber et al. (128) described the use of much shorter flashes (50 ms) which can be applied to measure F_M' more frequently with a reduced risk of photoinhibition or perturbation of electron transport.

77K Fluorescence Emission Spectra

A clear acrylic rod sample holder for 77K fluorescence was dipped briefly into the Hansatech PAM cuvette to collect about 100 μl; this subsample was plunged directly into liquid nitrogen. The sample holder was attached to a fiberoptic fluorometer, and 77K fluorescence emission spectra were collected (100), with excitation at 574 nm. The 77K spectra are therefore from cells under the same conditions as those used for room temperature fluorescence quenching and oxygen measurements.

Alternate Fluorescence Quenching Parameters

Havaux et al. (53) and Walters and Horton (152) have proposed the parameter $(1/F_O) - (1/F_M)$ as a measure of the rate constant of excitation trapping by the PS II reaction center and $1/F_M$ as a measure of nonphotochemical loss of excitation energy from the light-harvesting antennae. In our experiments, plots of $(1/F_O) - (1/F_M)$ closely parallel F_V/F_M. The $1/F_M$ parameter shows some discrepancy from cyanobacterial q_N and NPQ, possibly reflecting the distinct nature of nonphotochemical quenching in cyanobacteria and plants.

An alternate approach to extracting information from fluorescence signals is the pump and probe method reviewed by Falkowski and Kolber (40). For methodological and theoretical reasons, these authors define an alternate quantum efficiency term,

$$\Delta\phi_{\text{sat}} = (F_{\text{sat}} - F_{\text{probe}})/F_{\text{probe}}$$

as the difference in fluorescence yield driven by a weak probe light before and immediately following a saturating flash, divided by the fluorescence yield before the flash. This alternate parameter can be related to F_V/F_M as

$$F_V/F_M = \Delta\phi_{\text{sat}}/(\Delta\phi_{\text{sat}} + 1)$$

Another approach developed particularly for oceanographic purposes is fast repetition rate fluorescence (40), which uses a rapid train of 20 to 60 brief (5-μs), nonsaturating pulses applied at frequencies up to 200 kHz. Fluorescence excited by the first flash gives the F_O level fluorescence with all reaction centers open. The rapid succession of flashes then progressively closes PS II centers, driving fluorescence toward F_M. The rate of saturation is proportional to the effective absorption cross-section of PS II, σPS II, a useful parameter which is not determined by methods using single saturating pulses. The F_M measured with this train of pulses is not equivalent to the F_M determined with a single saturating pulse used in the modulated fluorometer (127, 128) or the pump and probe methods. Therefore, data from fast repetition rate fluorescence are not directly comparable or convertible to data from the other methods. Nevertheless, the results generate conclusions which are qualitatively similar to those obtained from quenching analysis involving modulated fluorescence (127).

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