Journal of Cellular Biochemistry 23:131–158 (1983)
Biosynthesis of the Photosynthetic Apparatus:
Molecular Biology, Development and Regulation 59–86

Fluorescence Decay Kinetics of Chlorophyll in Photosynthetic Membranes

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The absorption of light by the pigments of photosynthetic organisms results in electronic excitation that provides the energy to drive the energy-storing light reactions. A small fraction of this excitation gives rise to fluorescence emission, which serves as a sensitive probe of the energetics and kinetics of the excited states. The wavelength dependence of the excitation and emission spectra can be used to characterize the nature of the absorbing and fluorescing molecules and to monitor the process of sensitization of the excitation transfer from one pigment to another. This excitation transfer process can also be followed by the progressive depolarization of the emitted radiation. Using time-resolved fluorescence rise and decay kinetics, measurements of these processes can now be characterized to as short as a few picoseconds. Typically, excitation transfer among the antenna or light harvesting pigments occurs within 100 psec, whereupon the excitation has reached a photosynthetic reaction center capable of initiating electron transport. When this trap is functional and capable of charge separation, the fluorescence intensity is quenched and only rapidly decaying kinetic components resulting from the loss of excitation in transit in the antenna pigment bed are observed. When the reaction centers are blocked or saturated by high light intensities, the photochemical quenching is relieved, the fluorescence intensity rises severalfold, and an additional slower decay component appears and eventually dominates the decay kinetics. This slower (1–2 nsec) decay results from initial charge separation followed by recombination in the blocked reaction centers and repopulation of the excited electronic state, leading to a rapid delayed fluorescence component that is the origin of variable fluorescence. Recent growth in the literature in this area is reviewed here, with an emphasis on new information obtained on excitation transfer, trapping, and communication between different portions of the photosynthetic membranes.

Key words: chlorophyll, fluorescence lifetimes, photosynthesis, excitation transfer

The fluorescence of chlorophyll and other pigments provides a direct measure of the excited electronic states that result from the absorption of light by photosynthetic organisms. The kinetics of rise and decay of fluorescence intensity is a conse-

Abbreviations used: PS 1, PS 2, photosystem 1, 2; Chl, chlorophyll; PE, phycoerythrin; PC, phycocyanin; APC, allophycocyanin; DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethyl urea.

Received June 2, 1983; accepted August 11, 1983.

quence of an extensive series of excitation transfer steps followed by trapping in the photosynthetic reaction centers. The overall efficiency of conversion of absorbed photons into electron transfer reactions can be quite high; quantum yields of 90% or more have been reported. Of the excitation that is lost from the photochemical path, a significant portion appears as fluorescence of chlorophyll. This fluorescence is a valuable indication of the mechanisms involved in excitation transfer and trapping; it also reflects indirectly the efficiency of the photoactivation processes.

Chlorophyll *a* in simple solution in an organic solvent exhibits a fluorescence quantum yield of typically 30–40% [1]. Corresponding values measured for chlorophyll fluorescence in vivo are less than one-tenth of that value [1,2], which is entirely consistent with values of 90% or more for photochemical yields. The light reactions of photosynthesis exhibit saturation at high light intensities, and these light reactions can be blocked by the addition of inhibitors of electron transport. This decrease in the photochemical yield is accompanied by a complementary increase in the fluorescence yield, typically three- to fivefold. Duysens and Sweers [3] studied the transient responses to changes in excitation wavelength and concluded that the fluorescence yield is controlled by the state of reduction of the electron acceptor, Q, of photosystem 2 (PS 2). This variable component of the fluorescence, F_v, has been one of the tools most widely used to explore the primary photosynthetic light reactions. Variable fluorescence is observed from a variety of photosynthetic bacteria and from photosystem 2 (PS 2) of higher plants and algae, but it has not been detected unambiguously from PS 1. Several extensive reviews of the use of fluorescence of photosynthetic organisms have appeared in recent years [4–6].

By contrast with the fluorescence of a simple solution of chlorophyll, the emission of fluorescence from in vivo photosynthetic systems is not homogeneous in its origin. Several lines of investigation provide evidence to support this heterogeneity. (1) In addition to the variable fluorescence of chloroplasts, there is a background fluorescence, F_0, that is insensitive to the photochemical state of the reaction centers [1,2]. The level of F_0 is too large to be consistent with both a high photochemical quantum yield at low excitation intensities and a homogeneous fluorescence origin. (2) The wavelength dependence of both excitation and emission points to a heterogeneous origin. In the case of red algae and cyanobacteria, for example, the phycobilin accessory pigments have absorption and emission spectra that are distinctively different from those of chlorophyll [7]. There is also evidence for heterogeneity in green plants and algae that exhibit fluorescence only from chlorophyll *a*. This is particularly striking for measurements made at temperatures below about 200° K where several new peaks are observed in the emission spectra [8–10], but it is also evident from careful wavelength-dependence studies at room temperature. (3) Detailed investigations of the kinetics of the appearance of F_v upon turning on an actinic light are suggestive of two distinct origins of F_v, in α- and β-centers of PS 2 [11]. (4) Added components such as divalent ions [12] or changes in the level of phosphorylation of thylakoid membrane protein [13,14] alter the fluorescence emission in a fashion that reflects a heterogeneous origin. It is generally assumed that the different fluorescence components arise from different portions of the antenna pigment complement or from the reaction centers themselves. By and large, the evidence to support this assumption is lacking.

The complexity of the network of deexcitation paths available in vivo has complicated the analysis and interpretation of chlorophyll fluorescence in photosyn-

thetic organisms. This analysis has been rendered particularly difficult by the fact that the different fluorescence components present in vivo exhibit emission spectra that are commonly almost precisely superimposable on one another. In such cases, attempts to resolve the components on the basis of a distinctive wavelength dependence have been largely frustrated.

The kinetics of fluorescence decay provides a measure of fluorescence heterogeneity, even where the emission spectra overlap completely. Furthermore, the decay kinetics reflects directly the evolution with time of the excited states that result not only in fluorescence but also in the photochemical light reactions of photosynthesis. Interest in the path of this excitation flow and in the mechanisms of excitation transfer, trapping, and even the repopulation of excited electronic states has motivated a number of investigations of fluorescence decay kinetics extending now to times as short as 10 psec.

In this article we will review the measurements of fluorescence decay kinetics in photosynthesis, which began over 25 yr ago. Our emphasis will be on work during the past decade when fast time resolution became possible as a consequence of the widespread use of mode-locked lasers, streak cameras, and single-photon timing detection systems. We will somewhat arbitrarily restrict our attention to the investigations of chlorophyll *a*-containing, oxygen-evolving photosynthetic organisms. Fewer reports of fluorescence decay of photosynthetic bacteria have appeared; however, some significant advances have been made in those investigations. Also, we will survey only those aspects of "prompt" fluorescence that result in decays of 10 nsec or shorter. The very interesting and complex phenomena of delayed fluorescence have been reviewed elsewhere [6,15,16].

FLUORESCENCE DECAY KINETICS

The first measurements of fluorescence decay kinetics from photosynthetic organisms were conducted using flashlamp excitation and detection directly from the time response of a photomultiplier [17,18], or by phase-shift measurements using time-modulated excitation [19]. Even with the limited time resolution available, it was evident that the decay kinetics of chlorophyll was predominantly 1 nsec or shorter in vivo, compared with 5–6 nsec for the same molecule extracted into a simple solution. This observation was generally consistent with the expectation, based on the well-documented properties of homogeneous populations of fluorescing molecules, that there should be a linear correlation between fluorescence yield and fluorescence lifetime. In this regard the efficient photoinduced electron transport reactions of photosynthesis constitute quenching processes that shorten the excited state lifetimes.

Earlier investigations, especially by Duysens [7], had shown that the photosynthetic accessory pigments serve as sensitizers in stimulating excitation transfer to chlorophyll and to the reaction centers. Time-resolved studies by Tomita and Rabinowitch [20] indicated that these transfers occur within 0.3–0.5 nsec among the phycobilins of cyanobacteria and red algae, based on excitation and emission wavelength resolution of the fluorescence decay kinetics. Initial attempts to find evidence for multiple decay kinetics in green algae or plant chloroplasts were unsuccessful [21].

Tumerman and Sorokin [22] and Müller et al [21] used sensitive phase-shift techniques to explore the relation between the intensity of excitation and the observed

(average) fluorescence lifetime of chlorophyll fluorescence from PS 2 of green algae and plant chloroplasts. They observed an increase of 3–4-fold that correlated well with the concomitant increase in fluorescence yield. The fluorescence decay kinetics for these organisms was reported to be essentially independent of emission wavelength, and no evidence for multiple decay kinetics could be detected by using measurements at two different modulation frequencies [21]. In general, when only a single modulation frequency is used, the phase-shift technique gives only a single weighted mean lifetime even when multiple decay components are present. Several authors have discussed the consequences of the dependence of this mean lifetime on modulation frequency [2,21–23]. Studies by Moya [24] using the phase-shift method applied during the course of fluorescence induction in Chlorella pyrenoidosa or spinach chloroplasts detected curvature in the plots of fluorescence yield against lifetime. These results were interpreted in terms of a heterogeneous origin of the fluorescence. Borisov and Il'ina [25,26] applied the phase shift technique to digitonin-solubilized PS 1 and PS 2 particles in an attempt to detect contributions to the fluorescence originating in PS 1. Based on an indirect interpretation of their measurements, they concluded that the fluorescence lifetime component attributable to trapping by the PS 1 reaction center must be shorter than 30 psec [26].

The application to photosynthetic systems of very short (10 psec) pulses generated by mode-locked lasers beginning about 10 yr ago provided the first opportunity to obtain resolved fluorescence decays in the picosecond time domain. Detection was accomplished initially by means of a synchronized Kerr shutter [27] or a streak camera [28–30]. The initial studies were complicated by multiple-excitation phenomena associated with the very high photon fluxes required to obtain sufficient signal intensity to detect. This problem was recognized by Mauzerall [31] and by Campillo et al [32], and led to a series of studies in which the consequences of excitation annihilation were used to explore the rate and extent of excitation migration in the photosynthetic antenna pigment beds and to study the formation and decay of metastable quenching (triplet) states. These studies were well reviewed by Campillo and Shapiro [33] and by Breton and Geacintov [34], and they will be mentioned here only in passing.

The early mode-locked laser pulse results did show that the fluorescence rise in chloroplasts occurs very rapidly (< 10 psec) [27]; there was no evidence for an induction arising from a requirement of prior excitation transfer even when short wavelength (530 nm) exciting light was used. In most cases, however, the decay times observed were only a few hundred picoseconds or less, and these included contributions from biexcitonic annihilation processes when pulse energies of 10^{13} photons cm^{-2} or greater were used. The introduction of single-photon timing techniques permitted the use of much lower pulse energies (10^{6}–10^{11} photons cm^{-2}) coupled with the ability to resolve multiple decay components [35,36]. The first investigations used repetitive spark discharges that provide light pulses of a few nanoseconds duration, which limited the resolution of fast-decaying components. Subsequently, continuously pulsing mode-locked lasers that provide trains of 10–15 psec pulses at frequencies up to 10^{8} Hz have enabled the single photon-timing techniques to resolve components faster than 100 psec, even in the presence of large slow-decay contributions [37,38]. Recently, the development of accurately synchro-

nized streak cameras has permitted the use of lower laser pulse energies coupled with improvements in signal quality by averaging techniques [39,40].

In all of these methods it is difficult or impossible to resolve multiple exponential components when the decay times are closer than a factor of 3 to one another. Several groups have reported the detection of three decay components in the 0.05–3 nsec time range [41–43], but there are probably more contributions that will need to be extracted by other kinds of studies.

In addition to the search for fast-decay components associated with photosystem 1, the characteristics of the large increase in fluorescence at long wavelength (F730) that arises at low temperature ($< 200°$ K) in chloroplasts and algae have been studied. Using the phase-shift method, Butler and Norris [9] showed that F730 is associated with a relatively slow (3 nsec) decay. The application of fast time resolution methods led to the observation of a rising phase or induction of about 100 psec in the F730 emission when it is excited by short-wavelength radiation [44–47]. This is the first example in higher plant chloroplasts or green algae of a resolved fluorescence induction that probably reflects excitation transfer rates among the antenna pigments. Undoubtedly these inductions are present in the room temperature fluorescence as well, but the near identity of the emission spectra of all of the fluorescence components has made it impossible to distinguish fluorescence induction phases when they are superimposed by rapid decay components.

In the next sections we shall explore the aspects of fluorescence decay kinetics associated with PS 2, PS 1, and the light-harvesting pigments in more detail. It is important to keep in mind, however, that this review covers a period in which technology in this field has changed dramatically. Some of the authors' original conclusions were affected by instrument limitations and need to be reassessed. It is a tribute to the experimental skills of Brody and Rabinowitch [18] and Dmetrievsky, Ermolaev, and Terenin [19] that no major reassessment is needed for their pioneering work in this field that was reported in 1957.

EXCITATION TRANSFER AND TRAPPING

The light reactions of photosynthesis include light absorption by an antenna of chlorophyll and other pigment molecules, excitation transfer to a photochemical reaction center, and the initiation of electron transfer within the reaction center [48]. The primary chromophores responsible for light absorption in higher plants and algae are in an antenna of chlorophyll *a* molecules. In addition, accessory pigments such as chlorophyll *b*, carotenoids, and phycobilins extend the spectral region for the harvesting of light with subsequent energy transfer to chlorophyll *a*. Excitation transfer by exciton coupling within the pigment-protein complexes and by resonance or Förster transfer to neighboring antenna molecules is rapid, occurring within a few hundred picoseconds [49], and it terminates upon excitation trapping at the photochemical reaction center. The lower energy of the excited singlet state of the reaction center chlorophyll molecule and the possibility for delocalization of excitation among coupled molecules in the reaction center complex promote trapping [49]. In the excited state of the reaction center chlorophyll, weakly bound π-electrons in the conjugated porphyrin ring system permit efficient electron transfer to the primary electron

acceptor. Further electron transfer among donors and acceptors in the reaction center stabilizes the initial charge separation by decreasing the electrostatic interaction between the oppositely charged species.

As excitation migrates within the light-harvesting pigment-protein complexes, alternative excitation decay modes, such as fluorescence, compete with reaction center trapping. The efficiency of such competing reactions is correlated with the photochemical ability of reaction centers to carry out primary electron transfer. Consequently, the photochemical state of reaction centers is strongly correlated with the fluorescence yields and lifetimes of the associated chlorophyll molecules [3,21]. The open state, characterized by reaction centers capable of carrying out photochemistry, is associated with a low fluorescence yield and short fluorescence lifetime, while the closed state, exhibited by reaction centers where photochemistry is not possible, is accompanied by a high fluorescence yield and long fluorescence lifetime. Charge recombination between the oxidized primary electron donor and the reduced primary electron acceptor repopulates the excited singlet state of the reaction center chlorophyll, which results in the transfer of some of the excitation back to the chlorophyll antenna molecules. The possibility for continued migration of excitation in the light-harvesting antenna until another open reaction center is encountered depends upon the degree of communication between reaction centers. Various models of the photosynthetic unit address the fundamental questions of the degree of independence of the reaction centers and the uniformity of distribution of the light-harvesting chlorophyll molecules [50]. The simplest model [51–53] of the organization of photosynthetic units, the "puddle" or "separate package" model, envisions isolated reaction centers each with its own antenna. Photosynthetic units in a separate package arrangement would show decay components of the fluorescence with distinct lifetimes, one for units with open reaction centers and another lifetime for units with closed reaction centers. At the other extreme, the "lake" or "matrix" model pictures communicating reaction centers dispersed uniformly in a continuous array of antennae. Communication via excitation transfer between photosynthetic units is implied by the existence of antennae serving more than one reaction center. In a matrix arrangement there should be a continuous increase in the fluorescence lifetime as the reaction centers are progressively closed.

Experimental data also support intermediate degrees of cooperation between photosynthetic units. Melis and Homann [11] characterized the heterogeneity of PS 2 reaction centers in terms of α- and β-centers. A functional differentiation of the photosynthetic units is implied as a consequence of both a spatial separation on the thylakoid membranes and differences in the kinetic properties of photochemical energy conversion and charge stabilization of the two types of PS 2 reaction centers. α-Centers are characterized as PS 2 reaction centers randomly embedded in an array of chlorophyll *a/b* light-harvesting antenna and thus capable of communication via excitation transfer. The β-centers are isolated PS 2 reaction centers, each with its own chlorophyll *a/b* antenna complex and thus constituting a separate unit in terms of excitation transfer. The hypothesis of α- and β-centers combines aspects of the lake and puddle models, respectively.

The primary pigments that fluoresce are the chlorophyll *a* molecules. The accessory photosynthetic pigments of higher plants and green algae, chlorophyll *b* and carotenoids, contribute to the chlorophyll *a* fluorescence by the mechanism of radiationless inductive resonance (Förster) transfer to the light-harvesting chloro-

phyll. The accessory pigments phycoerythrin, phycocyanin, and allophycocyanin in blue-green and red algae have distinctive emission spectra, and fluorescence is observed from these pigments as well as from the chlorophyll [4]. The chlorophyll fluorescence seen in chloroplasts at room temperature is associated almost exclusively with PS 2, with weak contributions from PS 1 [3,54].

PHOTOSYSTEM 2 FLUORESCENCE

Origin of Variable Fluorescence

Duysens and Sweers [3] proposed that variations in the redox state of the quinone electron acceptor Q of the PS 2 reaction center can account for changes in the chlorophyll fluorescence yield. The oxidized Q in an open reaction center traps an exciton and thus quenches fluorescence; the fluorescence associated with open reaction centers is at a minimum level, F_0. As the acceptor Q is reduced, the reaction center becomes closed to photochemistry, and the fluorescence yield increases to a maximum level, F_{max}, when all of the reaction centers are closed.

A number of sources have been suggested for the origin of the F_0 level of fluorescence in open PS 2 reaction centers and the variable fluorescence in closed PS 2 reaction centers. Studies of the chlorophyll fluorescence decay kinetics in chloroplasts and green algae have led to a more conclusive determination of the origin of these fluorescence components. A recent review by Breton and Geacintov [34] summarized the early results reported for the chlorophyll fluorescence decay times of dark-adapted chloroplasts and Chlorella using excitation pulse intensities (greater than 10^{12} photons cm^{-2}) with potential exciton annihilation effects. The fluorescence decays of broken or intact chloroplasts of higher plants at room temperature using low excitation intensities (10^6–10^{11} photons cm^{-2}) are generally characterized by average lifetimes in the 0.2–0.8 nsec range. Improvements in the time resolution of single-photon counting systems with low excitation pulse energies ($\leqslant 10^{11}$ photons cm^{-2}) have led to recent analyses supporting triple exponential character for the chlorophyll fluorescence decay in chloroplasts and green algae [41–43,55]. Attempts to explain the multiexponential character of the chlorophyll fluorescence decay kinetics have prompted numerous studies investigating the effect on fluorescence kinetics of such factors as excitation intensity, inhibitor presence, reduction potential, method of subchloroplast preparation, and temperature. The following discussion reviews these experimental aspects of PS 2 chlorophyll fluorescence studies.

Intensity Dependence

Haehnel et al [41] investigated the kinetics of the chlorophyll *a* fluorescence emission in broken chloroplasts from spinach and peas and in the algae Chlorella *pyrenoidosa* and *Chlamydomonas reinhardtii* and found the fluorescence decay to be best described by three exponential components in all species. In spinach chloroplasts incubated in Mg^{+2}-enriched buffer, for example, the fluorescence decay at low light intensity of open reaction centers is best characterized by decay components with lifetimes of approximately 100, 400, and 1,200 psec. The chlorophyll fluorescence decay of these reaction centers when they are closed by high light intensity is composed of lifetime components of approximately 100, 750, and 2,000 psec. The fourfold increase in total fluorescence yield upon closure of the reaction centers is modest in comparison to the 30-fold increase in the yield of the slow component. The

variable fluorescence is thus dominated by the contribution of the slow phase.

These results are consistent with a model in which the fluorescence of the fast and middle phases represents excitation lost in transit to the PS 2 reaction center from different antenna pigments. The fast phase is proposed to be emission from the chlorophyll *a* antenna closely coupled to the PS 2 reaction center, while the middle phase reflects emission from the light-harvesting chlorophyll *a/b* antenna. A contribution from the antenna of PS 1 may also be present in the fast phase. The origin of the slow phase is attributed to PS 2 fluorescence following charge separation in a closed reaction center ($P^*_{680}\ IQ^- \rightarrow P^+_{680}\ I^-Q^-$) and a subsequent radical pair recombination of the oxidized primary electron donor, $P_{680}{}^+$, and the reduced pheophytin primary electron acceptor, I^- [56,57]. Excitation arising from the repopulation of the excited singlet state of chlorophyll ($P^+_{680}\ I^-Q^- \rightarrow P^*_{680}\ IQ^-$) may be transferred from P^*_{680} to the antenna chlorophyll molecules and emitted as the slow decay component of fluorescence.

Effect of Inhibitors

A number of studies have focused on the interpretation of fluorescence decay profiles in the presence of photosynthetic inhibitors such as 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU). Addition of DCMU blocks photochemistry at the level of the PS 2 reaction centers, closing the reaction centers by preventing electron transfer beyond Q. Thus the addition of inhibitor offers another means of influencing the fluorescence decay kinetics of chloroplasts by altering the state of the PS 2 reaction center. In general, the mean lifetime of PS 2 chlorophyll fluorescence increases upon the addition of DCMU [22,35,58]. Qualitatively similar results have been obtained in recent triple-exponential analyses of the room-temperature fluorescence decay of chloroplasts in the presence of DCMU [41,43,55]. In the work of Magde et al [43] dark-adapted chloroplasts with open reaction centers exhibited triple-exponential fluorescence decays with lifetimes of 40, 200, and 700 psec. Closure of the reaction centers with DCMU and hydroxylamine increased the lifetimes of the middle and slow phases to 1,300 and 2,400 psec, respectively. The increase in the middle phase lifetime is a difference from the results of the light-intensity study of Haehnel and co-workers [41]. The contribution of the fast phase to the fluorescence yield is small for both open and closed reaction centers and is attributed to PS 1 fluorescence by Magde et al [43]. The increased lifetimes of the two slower components upon addition of inhibitor are interpreted [43] in terms of a model in which PS 2 α- and β-centers differ in terms of their time-resolved fluorescence emissions as a consequence of different-sized antennae and distinct environments. This model is in contrast to that of Haehnel et al [41] in which the slow decay component reflects the kinetics of charge recombination in the reaction center.

Recent single-photon timing measurements of Chlorella vulgaris and Chlamydomonas reinhardtii at low excitation intensities at room temperature by Haehnel and co-workers [55] were resolved by a triple exponential fluorescence decay for both open reaction centers of dark-adapted samples and closed reaction centers of samples preincubated with DCMU and hydroxylamine. The kinetic components with lifetimes of approximately τ_1 = 130 psec (F_0 and F_{max}), τ_2 = 500 psec (F_0) and 1,100 psec (F_{max}), and τ_3 = 1,400 psec (F_0) and 2,200 psec (F_{max}) are in good agreement with the results obtained in Berkeley. The results also confirmed previous observations that the slow phase is the major component of the variable fluorescence [36,41]. The

TABLE I. Summary of the Decay Components of the Fluorescence Kinetics in Chloroplasts and Algae

	Fluorescence decay components			
	τ (psec)			
Species (conditions)	F_0	F_{max}	Origin	Ref
Spinach chloroplasts (5 mM Mg^{+2})	1,200	2,000	Chl *a*, following charge separation in a closed PS 2 reaction center and subsequent radical pair recombination of P_{680}^{+} and I^{-} (source of variable fluorescence)	[41]
	420	750	Light-harvesting Chl *a/b* antenna (possibly heterogeneous)	
	110	50	Chl *a* antenna (PS 1 and PS 2)	
Spinach chloroplasts (no Mg^{+2})	700	2,400	α- and/or β-PS 2 units	[43]
	200	1,300	α- and/or β-PS 2 units	
	40	40	Chl *a* of PS 1	
Chlorella vulgaris	1,400	2,200	Chl *a* of closed PS 2 reaction centers	[55]
	500	1,200	Light-harvesting Chl *a/b* antenna	
	130	100	Chl *a* of open PS 2 reaction centers	
Chlamydomonas reinhardtii	1,400	2,300	Chl *a* of closed PS reaction	[55]
	610	1,100	Light-harvesting Chl *a/b* antenna	
	110	60	Chl *a* of open PS 2 reaction centers	

effects of excitation and emission wavelength on the relative fluorescence yields of the kinetic components indicate that (1) the fast-decay component originates from chlorophyll antennae with an emission maximum at longer wavelength than that of the middle decay component, and (2) an excitation wavelength which is preferentially absorbed by chlorophyll *b* is favorable for exciting the chlorophyll antenna responsible for the middle decay component [55]. These results are consistent with the interpretation that the middle-phase decay originates from the light-harvesting chlorophyll *a/b* proteins and that the fluorescence of the fast and slow components is emitted preferentially by the chlorophyll *a* antennae of open and closed PS 2 reaction centers, respectively.

A summary of the decay components of the fluorescence kinetics in chloroplasts and algae as reported in these studies [41,43,45] is given in Table I.

Redox Dependence

The redox dependence of PS 2 fluorescence decay kinetics was investigated in the preliminary work of Sauer and Brewington [36] for spinach chloroplasts and in the work of Klimov, Shuvalov, and co-workers [38,56] for TSF II subchloroplast particles (discussed in the next section). A comprehensive potentiometric titration of the fluorescence decay kinetics in spinach chloroplasts at room temperature using low-intensity laser excitation and a picosecond-resolution single-photon counting

timing system has recently been reported by Karukstis and Sauer [59]. At all reduction potentials, poised with potassium ferricyanide and sodium dithionite, the fluorescence decay is best described by three exponential components [59] in agreement with the recent investigations of the PS 2 fluorescence decay kinetics as a function of illumination conditions and the presence of various inhibitors [41,43,55]. Furthermore, the biphasic character observed for the potentiometric titration curve of the PS 2 fluorescence decay kinetics agrees with the results of steady-state potentiometric titrations of total fluorescence yield [60–67]. As Q is reduced by lowering the potential, the slow phase changes 30-fold in yield with two distinct midpoint potentials of +119 mV and -350 mV at pH 8.0, accompanied by an increase in the lifetime of this phase from 1 to 3 nsec. The yield and lifetime of the fast (100 psec) component of the fluorescence decay are essentially insensitive to the state of the PS 2 reaction center. A small increase in the lifetime of the middle component (from 400 to 600 psec) occurs only during reduction of the high-potential component of Q.

The organizational differences ascribed to α- and β-centers are possible explanations for the functional heterogeneity observed for electron acceptor Q. The midpoint reduction potentials for Q_H and Q_L correlate well with those measured by Thielen and Van Gorkom [67] for the electron acceptors in β- and α-centers, respectively. However, the dependence of the slow-phase lifetime on the state of the reaction center during Q_H reduction is indicative of the presence of energy transfer between reaction centers, suggesting that Q_H is not equivalent to Q_β. Although the selective effect of reduction potential on particular fluorescence decay components provides further insight into the question of PS 2 organization and electron acceptor distribution, additional studies are needed for a conclusive interpretation of the observed PS 2 electron acceptor heterogeneity.

Photosystem 2 Subchloroplast Preparations

One promising means of interpreting the form of the chlorophyll fluorescence decay function in whole chloroplasts has been to study the chlorophyll fluorescence decay kinetics of PS 2 particles. In early work, Sauer and Brewington [36] found the fluorescence decay profiles of open (dark-adapted or ferricyanide-treated) or closed (dithionite- or DCMU-treated) D-10 PS 2 particles to be indistinguishable from those of unfractionated, digitonin-treated spinach chloroplasts. For open reaction centers in the D-10 particles, a single decay component of 0.34 nsec was observed, while for closed reaction centers two decay components of 0.8 nsec and 2 nsec were resolved. However, for chloroplasts not treated with digitonin, the chlorophyll fluorescence lifetime (0.2 nsec) of open reaction centers is less than that observed in the D-10 particles; and, similarly, in untreated chloroplasts with closed reaction centers, the magnitude and lifetime (0.48 nsec) of the long-lived component is less than that measured for D-10 particles. Treatment of chloroplasts with detergents such as digitonin thus has a measurable effect on the fluorescence lifetimes observed. This effect may arise as a consequence of an alteration in the physical organization of chlorophyll pigments in the subchloroplast particles. The detergent-induced kinetic differences of whole chloroplasts and PS 2 subchloroplast particles limit the usefulness of this method of analysis.

These results are qualitatively similar to the kinetics of fluorescence from TSF II particles measured by single-photon counting by Shuvalov et al [38]. The prompt

fluorescence lifetime was reported to be 0.42 nsec in the presence of 50 μM ferricyanide and 1.06 nsec in the dark at −450 mV. In addition, the low reduction potential induced the appearance of delayed fluorescence with a lifetime of 4.3 nsec, attributed to the radical-pair recombination of P_{680}^{+} and I^{-}. This delayed fluorescence is not observed, however, after reduction of pheophytin by continuous illumination at low potential.

Temperature Dependence

The temperature dependence of the fluorescence lifetimes monitored at emission wavelengths associated with PS 2 is generally observed to be small [45,47,68–70] and variable in the actual lifetime measured. A recent detailed study by Reisberg et al [47] used single-photon timing to measure the fluorescence decay components at wavelengths below 690 nm at 77°K in the presence of DCMU and Mg^{+2}. Three exponential components characterize the decay: a slow component with a lifetime of 1,500 psec (room temperature τ = 2,000 psec [41]), a middle component of 300 psec (room temperature τ = 600 psec), and a fast component with a lifetime of 150 psec (room temperature τ = 100 psec). The relative amplitude of the slow component decreases almost fourfold upon lowering the temperature to 77°K, while the middle-phase amplitude doubles and the fast component increases 1.5 times in amplitude. Consequently, the weighted mean lifetime of this decay component at 680 nm decreases from 1,200 psec at room temperature to 400 psec at 77°K. This weighted mean lifetime at 77°K is comparable to unresolved single-exponential measurements by the phase-delay method at 680 and 686 nm on pea chloroplasts of 420 psec by Wong et al [69] at 77°K. Furthermore, the slow and middle decay components [47] are in very good agreement with lifetimes of 0.5 nsec and 1.5 nsec resolved by phase fluorimetric methods in Chlorella at 77°K by Moya and Garcia [70] and attributed to emission from the PS 2 chlorophyll *a* antenna.

Avarmaa et al [45] extended the low-temperature fluorescence studies to the region of 4.2 to 77°K for PS 2 fractions of pea chloroplasts. The dependence of the fluorescence lifetimes of closed PS 2 reaction centers on emission wavelength (681, 685, and 696 nm) was interpreted to result from energy transfer between different chlorophyll forms. A tentative scheme of excitation energy transfer from the light-harvesting system of PS 2 (F681 and F685) to the reaction center antenna chlorophyll (F696) bound to P_{680} accounts for a decrease in fluorescence lifetime of the short-wavelength chlorophyll forms serving as excitation energy donors for the long-wavelength chlorophyll forms. For example, at 4.2°K the fluorescence lifetimes of F681, F685, and F696 were measured as 1, 2.5, and 4 nsec, respectively, demonstrating the greater efficiency of excitation energy transfer from the higher-energy (shorter-wavelength) chlorophyll forms. These results are in contrast to room-temperature fluorescence studies which assign fluorescence decay components to particular chlorophyll antenna complexes according to the principle that a shorter fluorescence lifetime is indicative of closer proximity to the PS 2 reaction center [41].

Progress in our understanding of the factors affecting the kinetics of PS 2 chlorophyll fluorescence is clearly limited by our lack of knowledge of the origin of such fluorescence. Complementary information about the organization of the PS 2 photosynthetic unit obtained by other biochemical and physical techniques is ulti-

mately necessary to develop a model of energy transfer consistent with measurement of PS 2 fluorescence decay kinetics.

PHOTOSYSTEM I FLUORESCENCE

Fluorescence attributable to PS 1 has proved more difficult to document than that from PS 2. There is no clear variable component associated with PS 1 reaction centers to serve as a handy diagnostic. In the absence of variable fluorescence, several alternative approaches to identifying PS 1 fluorescence have been developed; however, these are associated with some ambiguity or uncertainty in assignment. (1) Long-wavelength components of fluorescence were felt to be enriched in contributions from PS 1 because the PS 1 reaction center (P700) excited state lies at somewhat lower energy than does that of PS 2 (P680). The wavelength dependence of fluorescence decay kinetics is slight at room temperatures, however, and the long wavelength component is difficult to extract from the tail of the spectrum of the much larger fluorescence intensity from PS 2. (2) The fluorescence at low temperature, below about 200°K, from chloroplasts or green algae undergoes pronounced emission spectral changes. A prominent emission maximum appears at about 730 nm (F730) that can be more intense than F685 at 77°K. The species giving rise to F730 is clearly associated with PS 1; but the fluorescence intensity is not sensitive to the photochemical state of the PS 1 reaction centers, and F730 is even absent in some preparations that are highly enriched in P700. (3) The rise kinetics of F730 at low temperature has been used to monitor excitation transfer times from antenna pigments to PS 1. (4) The fastest fluorescence components at room temperature have been attributed to PS 1, often solely on the basis that the low fluorescence yield from PS 1 ought to be associated with rapid quenching. (5) Photosystem 1 particles or enriched fractions have been studied with the hope that elimination of PS 2 fluorescence would unmask the underlying contributions from PS 1. The detergent treatments used to prepare these particles have often resulted in the release of a portion of the chlorophyll into a "free pigment" form that is highly fluorescent and associated with a long lifetime (4–6 nsec) not seen in vivo. Even when formation of this free pigment does not occur, the particles always include a large amount of antenna chlorophyll along with the reaction center, and it is difficult to demonstrate that there has not been a partial disruption of the connections between the antenna and the reaction centers so as to alter the fluorescence properties.

Despite these difficulties and, in part, because of an awareness of them, some consensus about the properties of PS 1 fluorescence kinetics is beginning to emerge.

Long-Wavelength Fluorescence Associated With Photosystem 1 at Room Temperature

Early attempts to study the wavelength dependence of fluorescence decay kinetics from chloroplasts or green algae at room temperature were frustrated by the small differences involved [71] and, in retrospect, by the complexity of the decay process. Initial studies using high intensity mode-locked laser pulses and streak camera detection suffered from overriding fast decays attributable in part to exciton annihilation processes. Dramatic differences in both rise and decay kinetics for

chloroplast fluorescence depending on excitation and emission wavelength were reported [28,46], but these have failed to be confirmed in other investigations using low intensity excitation and other detection methods [41,72]. Using the phase-shift method, Moya et al [73] reported a small (ca 5%) decrease in mean fluorescence lifetime of spinach chloroplasts as the wavelength of emission was scanned from 680 to 740 nm; this may result from a PS 1 contribution. Possibly related are small wavelength-dependent effects on the relative amplitudes of the different components in resolved multiple exponential decays [43,55].

Low Temperature Fluorescence, F730

The dramatic increase in fluorescence of chloroplasts and green algae at low temperature has been attributed to pigments in proximity to PS 1. Using phase-shift measurements, Butler and Norris [9] showed that F730 of Chlorella pyrenoidosa at low temperature is associated with a relatively long decay time of about 3.1 nsec. This was attributed to a relatively small bed of antenna chlorophyll, C705, associated with PS 1.

Initial studies using high intensity (3.5×10^{14} photons cm^{-2} per pulse) mode-locked laser pulse excitation and streak camera detection resulted in appreciably shorter decay times ($\leqslant$ 1.1 nsec) for F730 [74,75]; intensity-dependence studies showed that this shortening arises from quenching processes, probably singlet-triplet exciton annihilation [76]. Subsequent studies using low intensity mode-locked laser excitation pulses and single-photon timing detection confirmed the long (3 nsec) decay time of F730 at 77°K and below [45,47].

More detailed studies of the wavelength dependence of the emission decay kinetics have revealed contributions from several long wavelength components with different decay times [46,70,73]. Overlap of these components and contributions from the long-wavelength tail of the emission from fluorescence associated with PS 2 leads to complicated decay patterns that have yet to be satisfactorily sorted out [47].

The long wavelength emission (F730) at low temperature does not exhibit any significant dependence on the state of the reaction centers of PS 1 [71,77], in contrast to the behavior of PS 2. Similarly, the presence or absence of salts ($MgCl_2$, NaCl) that affect the distribution of excitation between PS 2 and PS 1 has no effect on the lifetime of the major component of F730 [69,78].

Thus, the origin of these long-wavelength emissions at low temperature and their relation to PS 1 is not clear. Studies of postetiolated bean seedlings show that F730 has not appeared after 2 hr of illumination at room temperature [79]; it was seen after 28 hr of illumination for seedlings and was well developed, with a decay of 3 nsec. It is not known in detail how this appearance of F730 correlates with the rise in PS 1 activity, which appears and rapidly increases during the first few hours of illumination [80,81].

Do these long-wavelength emission components that are so prominent at low temperature also contribute significantly at room temperature? One way to approach this question is to examine the temperature dependence of F730 in sufficient detail to extract an activation energy for the process. Based on published values of 0.07 [46] and 0.08 eV [72] and assuming Arrhenius behavior, this component would contribute less than 10^{-3} as much fluorescence at room temperature and would not be detectable

at currently available sensitivities. This view is consistent with the fact that the distinctive, slow-decaying F730 component is not observed at temperatures above about 200°K [46,47].

Rise Kinetics of F730

An induction in the onset of the fluorescence rise of F730 at low temperature has been reported to require 20–150 psec [44–47,82]; in one additional study the onset was reported to occur in less than 50 psec [77]. This onset is not seen for F685 at low temperature, nor is it observed for the fluorescence from chloroplasts or green algae at room temperature. Where it was observed, the onset was attributed to excitation transfer from shorter-wavelength-absorbing chlorophylls that account for the absorption of most of the exciting light. It was noted that the decay kinetics of a portion of F685 at low temperature corresponds approximately to the rising phase of F730 [46,47]. Thus, F730 appears to result from excitation transfer to a small portion of the antenna, C705, associated with PS 1 when this transfer occurs at low temperature where the normal photochemistry is blocked. It is not known what role C705 plays at room temperature.

Fast Fluorescence Decay Components at Room Temperature

Studies of chloroplast fluorescence decay using high intensity (10^{13} to 10^{16} photons cm^{-2} per pulse) mode-locked laser pulses and streak camera or fast Kerr shutter detection showed fast decay components ($\leqslant 100$ psec) of considerable amplitude [28,29,74]. Although these were attributed to PS 1, it remained to be determined whether exciton annihilation (biphotonic) processes were contributing to the short decays. Several investigations indicated that the decay times and amplitudes are independent of excitation intensity when single pulses are used [76,83], but trains of pulses produce the buildup of triplet states that are highly quenching [72,76,84]. In studies using single pulses, the yield of fluorescence decreases strongly with increasing intensity (10^{12} to 10^{16} photons cm^{-2} per pulse) [85], which means that there must be a decrease in either the decay time or the amplitude, or both. At least one report noted an increase of the decay time (to 350 psec or greater) at intensities below 10^{13} photons cm^{-2} at room temperature [46].

Better time resolution using lower intensity excitation and either synchronized streak camera or single-photon timing detection has revealed that the fluorescence at room temperature exhibits at least three decay components at all wavelengths. Kushida et al [86] reported a fast (100 psec) decay in Chlorella vulgaris present in the emission at 700 nm, but not at 670 nm. Fast decays in the range 50–130 psec have been regularly detected using three-component resolution of the fluorescence from plant chloroplasts and green algae [41–43, 87], although the wavelength dependence observed by Kushida et al was not found for spinach chloroplasts [41]. Furthermore, this fast component continued to be present as the temperature was lowered to the vicinity of 100°K, albeit with progressively decreasing yield [47]. An estimate of the relative initial amplitude of the fast component at room temperature (70–80%) by Gulotty et al [42] led to the conclusion that not all of this fast fluorescence quenching occurs in PS 1. In other recent studies lower relative yields of the fast component were reported [41,43].

Photosystem 1-Enriched Particles

The expectation that PS 1 particles would exhibit enhancement of fast fluorescence decay components was fulfilled by early reports from Borisov and Il'ina [25,26] based on a somewhat indirect analysis of phase-shift measurements. These measurements and others using high-intensity laser pulse excitation [28,88] showed that the slower-decaying fluorescence components seen in intact chloroplasts were generally absent in PS 1-enriched particles. At the same time the fast decaying components were also observed in PS 2 particles, and it remained to be demonstrated that they were not produced by biphotonic processes resulting from the high excitation intensities. The first low-intensity measurements of fluorescence from PS 1 particles using single photon timing detection showed either an unresolved intermediate decay time (270 psec) [36] or evidence of both fast (110 psec) and slow (1,190 psec) components [37]. In all of these cases digitonin-solubilized particles were used, and these are known to give incomplete separation of PS 1 and PS 2. Low-temperature measurements on the digitonin PS 1 particles showed the presence of the slow decay kinetics (2–3 nsec) characteristic of F730 in intact chloroplasts [45]. Using wavelength-resolved phase-shift techniques, Moya and co-workers have carried out deconvolutions of the emission spectra of Triton-solublized PS 1 particles, and this has resulted in the detection of additional fluorescence contributions that had not previously been observed [73]. The long-wavelength, slower-decaying low-temperature emission from F720 (0.3 nsec) and F735 (2.5 nsec) was largely absent at room temperature, where the much weaker fluorescence emission was dominated by shorter decay contributions ($\leqslant$ 100 psec).

Recently new procedures have been developed to prepare more highly enriched PS 1 particles with lower ratios of chlorophyll to P700. Korvatovskii et al [89] studied a preparation containing 50 chlorophylls/P700 that exhibited no low-temperature, long-wavelength-enhanced fluorescence attributable to F730. Using high-intensity laser pulses and streak camera detection they found the fluorescence to be dominated by a 30-psec decay at room temperature; the decay time increased only to about 60 psec at 77°K. Slower components with decay times of 150 psec were also present, but the 3-nsec decay associated with F730 was not seen in these particles. The authors reported an effect of oxidizing $P700^+$ chemically at room temperature in increasing the decay time from 30 to 60 psec.

The most highly enriched PS 1 particles containing only 8–10 chlorophylls/P700 were prepared by a procedure of Ikegami [90]. Recently, the fluorescence decay kinetics of this preparation at room temperature was reported using high-intensity (10^{14} to 10^{18} photons cm^{-2}) excitation at 532 nm and streak camera detection [90]. Decay components of 10 and 70 psec and about 6 nsec were observed. The latter was observed to increase strongly upon heating the sample and was attributed to sample denaturation. Measurements at several emission wavelengths were indicative that the fastest (10 psec) component dominates the decay in the 690–700-nm region, whereas the 70 psec component results in emission primarily in the 670–680 nm region. It is not easy to reconcile these results with a simple mechanism of excitation transfer from short to long wavelength pigments; the authors attributed them to two independently fluorescing antenna components.

Clearly, much additional work needs to be done on the properties of the fluorescence associated with PS 1 before the nature of its contribution to the complex behavior of excitation migration and trapping in intact chloroplasts will be understood.

ANTENNA CHLOROPHYLL PROTEINS AND PHYCOBILINS

An essential function of the light-harvesting or antenna pigment proteins is to absorb radiation and transfer the resulting electronic excitation to a reaction center. The detailed mechanism of this excitation transfer is not known, but it probably involves a combination of rapid (subpicosecond) exciton delocalization among closely coupled chromophores within a single protein followed by slower (tens of picoseconds) Förster inductive resonance transfer between adjacent antenna complexes [92].

An important feature of the pattern of excitation migration is that it generally flows from chromophores in higher energy electronic states to those with lower energy excited states. Thus, excitation absorbed by carotenoids or phycobilin pigments at short wavelengths is transferred to chlorophyll, which has low energy absorption bands in the red region of the spectrum. There is a notable exception in the photosynthetic bacteria Rhodopseudomonas viridis, where energy clearly flows "uphill" with good efficiency from low-lying antenna states to a somewhat higher reaction center excited state. Although similar processes of uphill energy flow have not been well studied in higher plants, one should keep in mind the possibility of contributions from thermally activated excitation transfer.

The detailed process of excitation transfer among a complex set of antenna pigments has been studied for the phycobilins of cyanobacteria and red algae [7,93–95]. These organisms contain several phycobiliprotein pigments that are organized into carefully designed antenna complexes called phycobilisomes [96]. Duysens and others have shown that excitation of the shorter wavelength absorbing pigments results in the stimulation of fluorescence of the longer wavelength pigments, but not vice versa [7,93–95]. A representative pattern for red algae would be: phycoerythrin (PE) → phycocyanin (PC) → allophycocyanin (APC) → chlorophyll (Chl). If these transfer steps were highly efficient, one would expect to see fluorescence emission only from the terminal (Chl) pigment. That is not the case, however. The emission bands of the several pigments are resolved from one another well enough that the fluorescence of each component can be separately monitored. This proved to be an ideal situation for monitoring excitation transfer times using measurements of the kinetics of both the rise and decay of the stimulated fluorescence emission. Some of the earliest studies of fluorescence decay kinetics were carried out on phycobilisome-containing organisms [17,18,20]; although evidence of delayed excitation transfer was seen, the time resolution of the apparatus used was not sufficient to provide an accurate measure of the excitation transfer times.

The situation in higher plants and green algae is not so favorable for these studies. Much of the antenna pigment complex consists of chlorophyll *a* and chlorophyll *b* molecules, and no significant wavelength differences among the different fluorescence decay components at room temperature have been seen. While the strong overlap between absorption and emission bands of these higher plant antenna pigments is favorable for efficient excitation transfer, it does not permit the experimentalist to use different excitation and emission wavelengths to search for separate rise and

decay kinetic components. (A notable exception occurs at low temperatures, where the long-wavelength F730 emission associated with PS 1 has been observed to have an inductive rise attributable to excitation transfer from antenna pigments, as discussed in the previous section). Carotenoids are additional accessory pigments in higher plants and green algae, and detailed studies of the kinetics of the transfer of excitation from carotenoids to bacteriochlorophyll in photosynthetic bacteria have already proved rewarding. Similar investigations are needed for higher plants. The carotenoids themselves are not significantly fluorescent, even in the absence of excitation transfer, so measurements must be made of carotenoid-stimulated chlorophyll fluorescence. There is no carotenoid absorption wavelength where it is possible to completely avoid the simultaneous direct excitation of chlorophylls, but the differences are large enough that it should prove relatively easy to sort out the direct excitation from the stimulated process.

Red Algae and Cyanobacteria

Porphyridium cruentum is the only red alga where the fluorescence decay kinetics has been studied extensively in the intact state. Fluorescence decay from the chlorophyll *a* has been reported to have decay times ranging from 0.5 to 1.5 nsec at room temperature [18,20,23,97,98]. Techniques capable of resolving multiple exponential decays have not been applied to the Chl fluorescence from red algae. Therefore, it is generally assumed that excitation transfer occurs from the phycobilin accessory pigments to a homogeneous pool of fluorescing chlorophyll. This is likely to be a serious oversimplification, as has been found for higher plants and green algae.

Excitation transfer from the phycobilin chromophores to chlorophyll was measured extensively by Porter et al [99] by monitoring both the rise and decay kinetics at four wavelengths characteristic of the emission of PE, PC, APC, and Chl, respectively. Excitation at 530 nm was absorbed predominantly by PE, the fluorescence of which rose with no measureable induction (< 10 psec) and decayed nonexponentially in about 70 psec. The rising phases of PC, APC, and Chl fluorescence were delayed by 12, 24, and 50 psec, respectively, and this was taken to reflect the sequence of excitation transfer steps and to be indicative of the transfer times involved. The decays of the fluorescence of these pigments, observed to be exponential, were similarly delayed. The 175-psec decay time for Chl fluorescence in these studies is quite fast, despite the efforts of the authors to use laser pulse energies (10^{13} to 10^{14} photons cm^{-2}) as low as possible in their laser system. Apart from this difficulty, however, the results are in agreement with the pattern of excitation transfer proceeding sequentially from short-wavelength to long-wavelength absorbing pigments, as deduced by Duysens [7]. Lowering the temperature was found subsequently to increase the excitation transfer times somewhat and to increase the observed fluorescence decay times [100].

Similar studies of intact cyanobacteria have focused on the organism Anacystis nidulans. In general, the results are not qualitatively different from those of the red algae. Chl fluorescence decay times occur in the range 0.5–1.2 nsec at room temperature [18,20, 23, 98] and transfer times in the range of 0–75 psec [100,101]. The absence of PE in the pigment complement of Anacystis decreases the number of different chromophores involved in the transfer and similarly decreases the complex-

ity of the wavelength-dependent information compared with that of Porphyridium cruentum; however, in most respects the excitation transfer processes appear to be quite analogous in these two organisms.

Phycobiliproteins

The accessory pigments of cyanobacteria and red algae can be separated as monomeric or oligomeric phycobiliproteins or as aggregated heterocomplexes, known as phycobilisomes, that represent the organization of the phycobiliproteins in vivo. None of these pigment proteins contains Chl; however, there is a variety of phycobilin pigments that characterize the different phycobiliproteins. Some of the isolated phycobiliproteins, such as C-phycocyanin and C-phycoerythrin, contain only a single type of chromophore, whereas others like R-phycocyanin and phycoerythrocyanin contain more than one chromophore type [96]. The phycobiliproteins occur commonly as monomeric, trimeric, hexameric, and more highly aggregated species, and even the "monomers" typically contain several chromophores and two polypeptide subunits. Thus, excitation transfer contributes extensively to the excited state kinetic properties at almost all levels of organization.

Isolated phycobiliproteins are highly fluorescent, and measurements of fluorescence decays show exponential kinetics with lifetimes of 1.0–2.2 nsec for phycocyanins [102–107], 1.6–3.2 nsec for phycoerythrins [105–108], and 1.8–2.7 nsec for allophycocyanins [105,106,109], the longest-wavelength-emitting forms. In these isolated phycobiliproteins the rise of the fluorescence upon excitation is typically very fast (< 12 psec) [109], by contrast with the behavior of intact cyanobacteria or red algae as reported above.

Phycobilisomes

The isolation of intact phycobilisomes from Porphyridium cruentum led to a study of their fluorescence kinetics by Searle et al [110]. Excitation at wavelengths absorbed by B-PE led to a rapid rise and 70-psec decay of its fluorescence, essentially the same as the kinetics in the intact red algal cells [99]. Comparison with the fluorescence decay of isolated B-PE, where a fluorescence decay of 3.2 psec has been reported [105], is suggestive that excitation transfer from B-PE to R-PC and to APC occurs with 98% efficiency. Fluorescence from APC in the phycobilisomes exhibits decay kinetics of several nanoseconds, which is appreciably longer than in the intact cells, where excitation transfer to Chi serves an efficient quenching process. A more extensive study of phycobilisomes from the red alga Rhodella violacea was carried out by Holzwarth et al at low pulse energies using the single-photon timing method [111]. In addition to the rapid decays indicative of transfer from B-PE to C-PC (34 psec) and from C-PC to APC (25 psec), they observed a slow decay (2.1 ± 0.3 nsec) from the APC. This value is similar to that (2.7 nsec) reported for isolated APC from Porphyridium cruentum [105].

Phycobilisomes from cyanobacteria exhibit generally similar properties. Studies of the complexes isolated from Nostoc sp using pulse energies of 10^{14} photons cm^{-2} led to the observation of PE fluorescence decay of 31 psec and a nearly identical rise time for PC and APC fluorescence emission [112]. The final decay of APC fluorescence occurred in about 1.4 nsec at the lowest pulse energies (2×10^{13} photons cm^{-2}). Recent studies of the phycobilisomes from Mastigocladus laminosus showed that the fast-decaying component (70 psec) at 614 nm from PEC is strongly polarized

compared with the longer-wavelength emission from PC (150 psec) or APC (1.4 nsec) [40]. The decrease in polarization with increasing time during the decay process was attributed to the extensive excitation transfer that precedes the slower decay steps. There is likely to be little contribution from rotational diffusion to the fluorescence depolarization on this time scale in phycobilisomes.

These studies of phycobilisome fluorescence kinetics provide the clearest example to date of the dynamics of excitation transfer among a collection of closely associated but different chromophores. The fluorescence rise and decay studies show that (1) excitation transfer proceeds systematically from short-wavelength to long-wavelength chromophores; (2) only the very earliest fluorescence decay exhibits significant polarization; (3) the decay of the fluorescence of the initially excited, short-wavelength-absorbing chromophores coincides with the rise in fluorescence of the longer-wavelength forms; (4) fluorescence decay kinetics of the shorter-wavelength components in phycobilisomes is much shorter than that of the corresponding isolated phycobiliproteins, indicative of excitation transfer efficiencies of 90% to 98%; and (5) the terminal fluorescence in the excitation transfer process in the phycobilisomes fluoresces with a decay time that is nearly the same as that of isolated APC.

Light-Harvesting Chl *a/b* Protein

The only isolated antenna component from higher plants that has been extensively studied using fluorescence kinetics is the LH Chl *a/b* protein. High molecular weight forms of this antenna complex were reported to have fluorescence decay constants of 4 nsec using excitation pulses of 10^{14} photons cm^{-2} [113] and 2.5 nsec at 3×10^{12} photons cm^{-2} [114] with streak camera detection. In the latter study [114,115] the lifetime was found to decrease to 0.5 nsec at pulse energies of 10^{14} photons cm^{-2}; at the lowest energies the decay of fluorescence in the LH Chl *a/b* protein was 5–8 times slower than was the decay from PS 2 particles or chloroplasts.

Subsequent investigations using either the phase-shift technique [116] or a synchronized streak camera [39] showed that the measured lifetime is strongly dependent on the state of aggregation of this complex; however, the aggregated form gave rise to a significantly shorter decay, 0.6–1.2 nsec [39,116] than was reported initially. A solubilized monomeric form of the complex was found to have a longer decay, 4.1 nsec [116] or 3.1 nsec [39], but with no intensity dependence in the range from 10^{14} to 3×10^{18} photons cm^{-2} [39].

Using single-photon timing measurements, Lotshaw et al [117] found the monomeric species to exhibit biexponential decay, with $\tau_1 = 1.2$ nsec and $\tau_2 = 3.3$ nsec, occurring with about equal yields of the two components. No significant dependence on either the excitation or emission wavelength was noted in this investigation. Different results were obtained by Moya et al [73] using phase shift measurements. They found shorter decay times in measurements at room temperature, with a small dependence on emission wavelength: 0.77 nsec at 680 nm and 0.85 nsec at 700–720 nm. At 77°K a much more pronounced wavelength dependence, 0.4 nsec at 685 nm to 2.45 nsec at $\geqslant$700 nm, was found.

It is difficult at present to reconcile the results from different laboratories. The strong dependence of the lifetime on the degree of aggregation makes it essential for future investigators of these preparations to take particular care in characterizing their materials. It may be that the multiple exponential components observed by Lotshaw

et al [117] result from some heterogeneity in their sample, for example. Until this antenna complex is better understood, it will be difficult to speculate about its contribution to the excitation transfer and emission decay components of intact chloroplasts.

PHOTOSYNTHETIC MEMBRANE ORGANIZATION

A large majority of the chlorophyll molecules in photosynthetic membranes comprise the light-harvesting chlorophyll *a/b*-protein complex and the individual chlorophyll *a* antennae for the photochemical reaction centers [118,119]. The efficient transfer of energy absorbed by the antenna to the reaction centers is presumed to occur by the mechanism of singlet excitation, or exciton, transfer within a pigment-protein complex, and by resonance or Förster transfer between neighboring complexes. The coulombic interaction between the electrons of light-harvesting pigment molecules promotes the transfer of excitation. Thus, the rate of excitation transfer between antenna molecules is both distance- and orientation-dependent [52]. A simlifying terminology to describe the organization of pigments in the photosynthetic apparatus was introduced by Robinson [120] and developed by Knox [51–53]. Antenna pigments forming an extended array supplying excitons to many different reaction centers constitute a "lake" model, while independent photosynthetic units each with their own reaction center comprise a "puddle" model. More extensive models for the organization of the energy-trapping components of the photosynthetic unit include the continuous array model of Seely [121], the pebble mosaic model of Sauer [122], and the matrix or pigment bed model of Duysens [123]. Seely's model [121] regulates optimum electron transport by variations in chlorophyll molecule orientation. Irregularities in the arrangement of chlorophyll-protein complexes control the extent of electronic excitation energy transfer between photosynthetic units in Sauer's pebble mosaic model [122]. The photosynthetic units of the matrix model, however, are without boundaries for energy transfer [123]. In the tripartite model of Butler and Kitajima [124,125], the energy distribution within the photochemical apparatus is controlled by two parameters, the initial partitioning of quanta between the two photosystems and the subsequent transfer to PS 1 of some of the quanta initially delivered to PS 2 via the light-harvesting chlorophyll *a/b* antenna. A modification of this model by Butler [126] allows for direct energy transfer from PS 2 to PS 1 by the positioning of adjacent PS 2 and PS 1 units, with the light-harvesting chlorophyll associated with PS 2 and only in partial contact with PS 1. A comprehensive review of current models of the organization of photosynthetic pigments in vivo has been recently presented by Thornber and Barber [50].

Measurements of fluorescence decay kinetics and fluorescence risetimes have played an important part in the determination of energy transfer rates and in the development of a basic model for the photosynthetic apparatus. For example, the differing kinetics of the 735-nm PS 1 fluorescence rise and the 685-nm PS 2 fluorescence decay as measured at low temperature by Campillo et al [44] prompted the proposal of a division of the light-harvesting pigments into two types. One type of light-harvesting antenna is closely associated with PS 1 with unidirectional transfer to the PS 1 reactions centers within 150 psec. The remaining larger portion of light-harvesting pigments, not closely connected to PS 1 because of a possible spatial separation, transfers its energy to PS 2 within 800 psec. Excitation transfer of excitons

to the light-harvesting pigments of PS 1 is also possible but does not constitute a major pathway in this model.

Wavelength-resolved chlorophyll fluorescence emission studies do not allow for the possible discrimination between various models of the photosynthetic unit because the different antenna chlorophyll pigments of green algae and higher plants are not easily selectively excited, nor do they fluoresce in separate spectral regions at room temperature. As a consequence, Haehnel et al [55] investigated the relative effects of changing the excitation and emission wavelengths on the fluorescence yields of the kinetic decay components of Chlorella vulgaris and Chlamydomonas reinhardtii. Arguments presented ascribe a fast 100-psec decay component to the chlorophyll *a* antenna of PS 2 and a 400 to 750-psec component to the light-harvesting chlorophyll *a/b* antennae. This interpretation is in agreement with the tripartite model of Butler [124,125]. A similar model of the photosynthetic unit may be applicable to the results of the excitation-wavelength study of the fluorescence decay components in barley chloroplasts by Gulotty et al [42]; however, the reported amplitudes of the fluorescence decay components are not consistent with measurements of the relative distribution of chlorophyll pigments in PS 1 and PS 2 reaction centers and the chlorophyll *a/b* protein-complex.

Intersystem and Intrasystem Energy Transfer

The existence of unidirectional exciton transfer, or spillover, from PS 2 to PS 1 has been considered as a possible control process for optimizing energy distribution between the two photosystems [125,127–133]. Investigations involving fluorescence decay kinetics attempt to make a distinction between the particular level of cooperativity between the two photosystems—whether cooperation takes place directly at the level of intersystem energy transfer or indirectly via changes in the partitioning of absorbed quanta between the two photosystems. An investigation of Barber et al [132] of cation-induced changes in fluorescenece yields and lifetimes reported a marked decrease in the fluorescence decay rate and an increase in the steady-state fluorescence quantum yield upon addition of $MgCl_2$. They concluded that salt addition affects the distribution of energy between the photosystems by changing the rate of energy spillover from PS 2 to PS 1, rather than the partitioning of incident quanta between the light-harvesting chlorophylls of the two photosystems. Nairn et al [130] reported a similar decrease in the energy transfer or spillover from PS 2 to PS 1 upon $MgCl_2$ addition and further demonstrated that this effect saturates at low concentrations of Mg^{+2} (less than 0.75 mM). A second effect of divalent cation is reflected in a decrease in both the F_0 lifetime and yield of the slow phase of the fluorescence decay in the range of 0.5 to 2.0 mM Mg^{+2}. The proposed origin of this effect is an increase in both the absorption cross section and the extent of energy transfer between PS 2 units. Differences in the intensity dependence of the slow-phase lifetime in the presence and absence of added cation also reflect the extent of energy transfer between PS 2 units. This three-component analysis [130], in qualitative agreement with other Mg^{+2}-dependent fluorescence lifetime measurements which resolve one [134] or two components [68,132,135], thus suggests the presence of cation-controlled regulation of the distribution of energy between the two photosystems and the possibility for cation-induced intercommunication between PS 2 units. However, Melis and Ow [131] have recently presented evidence using fluorescence induction kinetic measure-

ments that a spillover of excitation from PS 2 to PS 1 cannot fully account for the lowering of the fluorescence yield in Mg^{+2}-free media.

P to S Fluorescence Decline

The fluorescence decay kinetics during the P to S transition of the fluorescence induction in intact chloroplasts, algal cells, and green plant tissues has been studied [136,137] to substantiate theories which ascribe the fluorescence decline to alterations in the distribution of quanta between the two photosystems [138]. During the P to S fluorescence decline, Nairn [137] observed the triple-exponential fluorescence decay to exhibit a 60% decrease in the total fluorescence yield, accounted for by a sixfold decrease in the yield of the slow decay component, and a 20% decrease in the lifetime of each decay phase. The results of Nairn [137] are suggestive that the effect of the P to S transition on energy distribution in the thylakoid membrane is a reversal of the Mg^{+2} effect. A structural change of the chloroplast membranes is induced by the Mg^{+2} efflux from the thylakoids as a consequence of the inward pumping of H^+ that is coupled to the light-initiated electron transport [139–144]. The proposed [137] reduction in the absorption cross section of PS 2 and the increase in the rate of spillover from PS 2 to PS 1 during the P to S decline parallels similar changes associated with the removal of Mg^{+2} [130]. Furthermore, the differences observed in the fluorescence lifetimes and yields for the corresponding decay components of the P and S states correlate well with those differences noted in the presence and absence of Mg^{+2} [130].

Malkin and co-workers [136] investigated the relation between average fluorescence lifetime and total fluorescence intensity during the P to S transition for a variety of higher plants. The degree of linearity between these two parameters has been correlated with the extent of communication between reaction centers, a linear relation being characteristic of a lake model of photosynthetic units with significant energy transfer [51–53]. Malkin's observations [136] of both linear and nonlinear correlations of the lifetime and intensity of fluorescence during the P to S transition of various plants imply a number of different organizations of the photosynthetic units and suggest that further analyses of this phenomenon are necessary to understand the mechanism of the light-induced slow fluorescence quenching.

Membrane Development

The relation of PS 2 fluorescence to the structure and composition of PS 2 units may be studied by fluorescence decay measurements at various stages in thylakoid development as determined by the mode of illumination during growth. Exposure of etiolated leaves to continuous light leads to the transformation of the etioplast to a normal granal chloroplast [145]. Under conditions of intermittent light (eg, 2 min light/118 min dark), however, the etioplast is converted to a protochloroplast—with unstacked primary thylakoids devoid of chlorophyll *b* and deficient in the light-harvesting chlorophyll *a/b* complex [145]. The protochloroplasts can be transformed to chloroplasts only after transfer of the plants to continuous light. The etioplast to chloroplast differentiation offers a means of monitoring the development of PS 2 units and correlating structural changes to variations in the kinetic parameters of fluorescence decay.

Van der Cammen and Goedheer [79,146,147] used phase-fluorimetric measurements of the fluorescence lifetime during the greening of etiolated bean and barley

leaves to derive information about PS 2 unit formation and development. In particular, fluorescence lifetime and yield variations were associated with changes in the degree of phototransformation of protochlorophyllide, energy transfer between pigments, and aggregation of pigment molecules. Furthermore, he observed fluorescence lifetimes allowed for the construction of a comprehensive scheme for the path of protochlorophyllide reduction in developing leaves.

We have monitored the fluorescence decay kinetics of pea etioplasts exposed to intermittent and continuous light to correlate the known structural and organizational changes associated with the etioplast to chloroplast differentiation with fluorescence lifetime and yield variations [148]. The incorporation of chlorophyll *a* into small, uniformly sized photosynthetic units upon illumination of etioplasts parallels the decrease in lifetime and amplitude of the slow component of the three-exponential fluorescence decay. The increase in photosynthetic unit size with the development of the chlorophyll *a/b* light-harvesting complex and the subsequent grana formation are reflected in a differentiation of the slow-decay kinetics for open (F_0) and closed (F_{max}) reaction centers and in the observed cation-induced fluorescence rise.

The structural organization of photosynthetic membrane complexes can also be defined by using specific mutations affecting identifiable components within the complex. Searle et al [135] studied the organization and excitation energy distribution differences of chloroplasts from wild-type barley and the barley mutant chlorina f-2, which lacks the light-harvesting chlorophyll *a/b*-protein complex. The two-exponential fluorescence decay of the wild-type barley chloroplasts is resolved into a short lifetime component of 140 psec attributed in part to the PS 1 chlorophyll *a* antenna in the stromal lamellae and in part to the light-harvesting/PS 2 pigment beds strongly coupled with PS 1 in the granal lamellae. The component of 600 psec (F_0) or 1,300 psec (F_{max}) in the presence of Mg^{+2} is ascribed to those light-harvesting/PS 2 pigment beds not coupled to the PS 1 antenna. In the case of the chlorina f-2 mutant the two components of approximately 50 psec and 194 psec (F_0) or 424 psec (F_{max}) are attributed to the separate emissions from the PS 1 and PS 2 antenna chlorophylls, respectively, in the absence of the light-harvesting complex. The marked increase in lifetime produced by the presence of the light-harvesting complex in the wild-type barley compared with the mutant barley is interpreted [135] in terms of an excitation retention time in the light-harvesting complex long enough to allow for some fluorescence emission, either as a result of the reversible transfer of excitation between the light-harvesting and PS 2 pigment beds or as a consequence of a retention within the light-harvesting pigment bed prior to irreversible transfer to the chlorophyll *a* antenna of PS 2. The results are, in general, supportive of Butler's tripartite model [113, 125]. We are also investigating the fluroescence decay kinetics of the chlorina f-2 mutant and other photosynthetic mutants to derive a viable model for the arrangement of the major components of the photosynthetic unit [149].

Fleming et al [150] recently presented preliminary data on the fluorescence decays obtained from genetic mutants of Chlamydomonas reinhardtii which lack specific components of the photosynthetic apparatus. Comparisons of the fluorescence decay properties of wild-type (WT 2137), chlorophyll *b*-deficient (DS521), photosystem 1-deficient (12-7), and photosystem 2-deficient (8-36) Chlamydomonas show differences in both the lifetimes and amplitudes of the three exponential decay components resolved. The presence of a short-lifetime component in both WT 2137 and DS521 but with different lifetimes of 100 psec and 44 psec, respectively, implies

that the 100-psec decay component represents photochemical trapping originating from both the chlorophyll *a/b* light-harvesting protein and the chlorophyll *a* antenna of each reaction center. A comparison of WT 2137 and the PS 1 mutant 12-7 revealed a significant decrease in amplitude and increase in lifetime of the fast-decay component in the PS 1 mutant, suggesting that the fast component is associated with energy transfer to both photosystems. An increase in lifetime of the middle decay component for both the PS 1 and PS 2 mutants with respect to the wild-type Chlamydomonas is interpreted to represent a decoupling of chlorophyll *a/b* protein from quenching reaction centers. Presumably this decoupling is also associated with the changes in lifetime of the slow decay component from 2,900 psec in wild-type to 2,000 psec in the PS 2 mutant. Preparation of double mutant cultures [150] will further test these ideas.

SUMMARY

Clear evidence for the heterogeneous origin of the fluorescence from photosynthetic membranes is seen in the time-resolved decay kinetics. In general, the background fluorescence, F_0, is associated with short lifetimes (<1 nsec); both the amplitude(s) and decay lifetime(s) are largely insensitive to the state of the PS 2 reaction centers, and these fluorescence components are thought to result from excitation lost in transit to the reaction centers and in competition with photochemical trapping. There is little evidence for the presence of significant portions of disconnected pigments with unusually long decay times in undamaged chloroplasts, algal cells or cyanobacteria.

The variable fluorescence, F_v, is associated with an amplitude that changes over a wide (15–20-fold) dynamic range in response to reduction of the PS 2 electron acceptor; however, the lifetime increases only modestly (1–2 nsec), if at all. The large changes in the yield of this slower fluorescence decay component are consistent with a high ($>95\%$) photochemical quantum yield for PS 2. The origin of the slower fluorescence decay is attributed to charge recombination in closed reaction centers leading to repopulation of the Chl-excited electronic state—a form of fast delayed fluorescence.

Long-lived ($\geqslant 3$ nsec) fluorescence decays occur only under nonphysiological conditions, such as for isolated antenna complexes, at low temperature, for deficient mutants, under strongly reducing conditions, or for partly denatured samples.

Excitation transfer among the light-harvesting pigments occurs within roughly 100 psec, by which time the excitation has traversed several hundred chromophores and reached a reaction center trap. Studies involving fluorescence rise kinetics, the rate of fluorescence depolarization, and the sensitization by excitation transfer among different chromophores in phycobilisomes all lead to the same conclusion.

Changes in the organization of the photosynthetic membranes can alter the extent of communication between nearby photosynthetic units, and such changes are reflected in the fluorescence kinetic behavior. Models still need to be developed to account adequately for this behavior.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to many of their colleagues who sent articles in press or manuscripts in preparation for publication. The research from the authors' laboratory was supported by the Director, Office of Energy Research,

Office of Basic Energy Sciences, Division of Biological Energy Conversion and Conservation of the Department of Energy, under contract DE-AC03-76SF00098, and by a grant from the National Science Foundation [PCM 82-10524]. One of us (K.K.) wishes to acknowledge support from a National Institutes of Health National Research Service Award [1F32 GM08617-02].

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