membrane (*3*). For hernandulcin, two possible binding sites are the C-1 carbonyl and C-1′ hydroxyl groups because the sweet taste of the compound is lost when these groups are modified (*21*). Since these two functional groups are located about 2.6 Å apart in the preferred conformation, this molecule closely fits the Shallenberger model (*22*) for sweet-tasting compounds. Thus, the structural simplicity of hernandulcin, the availability of a facile synthesis for it, and the intense sweetness it exhibits make it a target molecule for the further investigation of the relation between structure and sweetness.

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10. Hernandulcin showed infrared absorptions at 3479 cm^{-1} (0.002 to 0.005*M* in carbon tetrachloride) and 1644 cm^{-1} (film) for an intramolecularly bonded hydroxyl group and an α,β-unsaturated keto group, respectively. The latter signal was confirmed by an observed maximum in the ultraviolet spectrum in ethanol at 236 nm (ε, 7775). Prominent fragment ions in the high-resolution mass spectrum of hernandulcin were observed at *m/z* 110 ($C_7H_{10}O$), *m/z* 95 (C_6H_7O), and *m/z* 82 (C_5H_6O), which suggested that it is a monocyclic sesquiterpene.
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15. The reaction was performed by adding 6-methyl-5-hepten-2-one (8.5 mmol) to a cold (−15°C) mixture of 3-methyl-2-cyclohexen-1-one (8.5 mmol) and lithium diisopropylamide (8.5 mmol) in anhydrous tetrahydrofuran (15 ml). After the reaction mixture was stirred for 5 minutes, it was worked up in the usual manner; purification by column chromatography afforded (±)-hernandulcin (4.1 mmol) and (±)-epihernandulcin (0.2 mmol). This type of reaction has been reviewed [T. Mukaiyama, *Org. React.* **28**, 203 (1982)]. Whereas (±)-hernandulcin is sweet, (±)-epihernandulcin exhibits no sweet taste.
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21. Derivatives of (±)-hernandulcin in which the C-1 carbonyl group was reduced to an alcohol or the C-1′ hydroxyl group was acetylated were not sweet.
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Disk-to-Disk Transfer as the Rate-Limiting Step for Energy Flow in Phycobilisomes

Abstract. *A broadly tunable picosecond laser source and an ultrafast streak camera were used to measure temporally and spectrally resolved emission from intact phycobilisomes and from individual phycobiliproteins as a function of excitation wavelength. Both wild-type and mutant phycobilisomes of the unicellular cyanobacterium* Synechocystis *6701 were examined, as well as two biliproteins, R-phycoerythrin (240 kilodaltons, 34 bilins) and allophycocyanin (100 kilodaltons, 6 bilins). Measurements of intact phycobilisomes with known structural differences showed that the addition of an average of 1.6 phycoerythrin disks in the phycobilisome rod increased the overall energy transfer time by 30 ± 5 picoseconds. In the isolated phycobiliproteins the onset of emission was as prompt as that of a solution of rhodamine B laser dye and was independent of excitation wavelength. This imposes an upper limit of 8 picoseconds (instrument-limited) on the transfer time from "sensitizing" to "fluorescing" chromophores in these biliproteins. These results indicate that disk-to-disk transfer is the slowest energy transfer process in phycobilisomes and, in combination with previous structural analyses, show that with respect to energy transfer the lattice of approximately 625 light-harvesting chromophores in the* Synechocystis *6701 wild-type phycobilisome functions as a linear five-point array.*

Conversion of light energy to chemical potential in biological photosynthetic systems is accomplished in macromolecular complexes composed of polypeptides and pigment molecules. A general feature of these complexes is the presence of numerous chromophores ("antenna pigments"), which absorb light and transfer the excitation quantum to a special chlorophyll or bacteriochlorophyll molecule in the "reaction center" for subsequent conversion to electron flow (*1*). Such complexes often contain several hundred antenna chromophores per reaction center. Since the overall quantum efficiency for the transfer of energy from the antenna to the reaction center is typically ~90 percent (*1*), energy transfer to the reaction center must compete successfully with pathways of energy dissipation in the antenna, such as fluorescence and nonradiative decay. Because most light-harvesting complexes are integral membrane components, information on the details of their molecular architecture is limited (*2*). Consequently, the structural basis for the highly efficient transfer of energy in these systems is not understood. However, an opportunity to correlate the structure and function of one light-harvesting system is provided by the phycobilisome, an antenna complex that efficiently funnels light energy collected over a broad spectral region and a large spatial area to the reaction center of photosystem II in cyanobacteria and red algae (*3*).

Phycobilisomes are peripheral membrane complexes that can be readily isolated without perturbing their structural and functional properties (*3*). Phycobilisome morphology depends on organismal origin. These particles have molecular weights from 7×10^6 to 15×10^6, contain between 300 and 800 tetrapyrrole (bilin) chromophores, and absorb light

over much of the visible spectrum. Energy absorbed by any of these chromophores is efficiently transferred to terminal energy acceptors in the particle via energetically favorable radiationless pathways (*3*). Phycobilisomes consist of rods made of stacked disks that radiate from a central core. They are constructed of phycobiliproteins, a family of brilliantly colored, water-soluble, oligomeric proteins that carry various covalently attached bilin prosthetic groups (*4, 5*).

The structures of phycobilisomes from *Synechocystis* 6701 and from the mutant strain CM25 (*6*) are diagramed in Fig. 1. The structures of the two particles are identical except that the phycoerythrin complexes that form the outer portions of the rod subassemblies are absent in the mutant phycobilisome. Also shown in Fig. 1 are the major species involved in the energy transfer pathway in *Synechocystis* 6701 phycobilisomes. Previous studies of *Synechocystis* 6701 wild-type phycobilisomes (*4–6*) have indicated that if each disk is considered as a single chromophoric unit, a maximum of five disk-to-disk transfers is required to convey the excitation energy to the terminal emitters: PE(30.5K) → PE(31.5K) → PC(33.5K) → PC(27K) → allophycocyanin complexes → terminal acceptors, where PE(30.5K), PE(31.5K), and so forth refer to disk complexes that make up the rod substructures of the phycobilisome (Fig. 1).

The phycobiliprotein complexes that make up the individual disks have fluorescence lifetimes of ~2 nsec (*7–9*). For making a rough calculation, we assume that all the transfers are equally efficient. For a five-step process, the 90 percent overall energy transfer efficiency observed (*10*) would require a 98 percent quantum transfer efficiency at each step. Given that each transfer competes with a fluorescence lifetime of ~2 nsec, a transfer efficiency of 98 percent implies that the overall time constant for the disk-to-disk transfer process must be ~40 psec.

To test the validity of this estimate, we

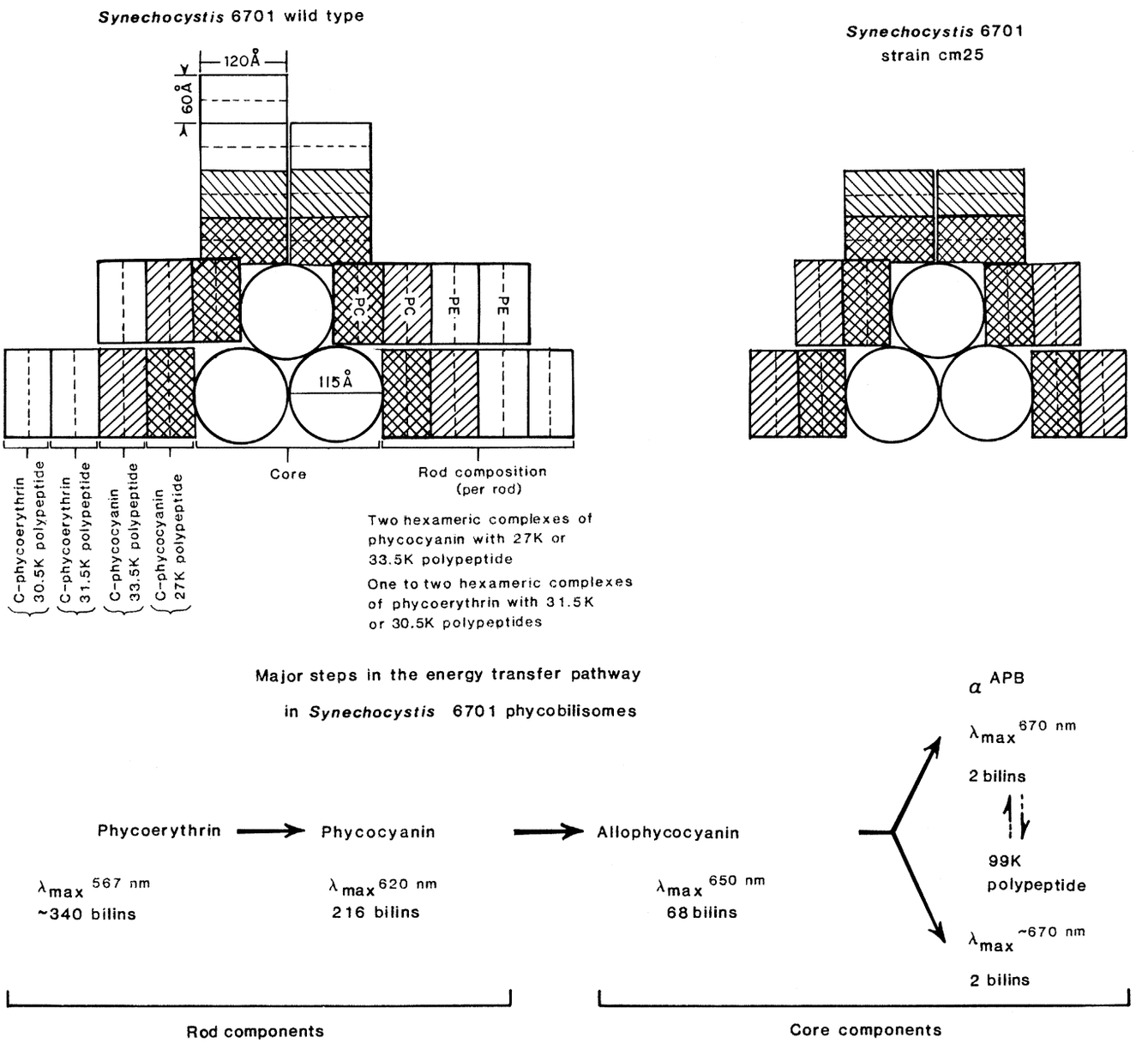

Fig. 1. Diagrammatic representation of the structure of *Synechocystis* 6701 and mutant strain CM25 phycobilisomes. Each disk is a hexameric biliprotein aggregate held together and joined to its nearest neighbor proximal to the core by a linker polypeptide, X, where X = 30.5K, 31.5K, and so forth. Thus the composition of each disk is $(\alpha\beta)_6X$. The number of bilins associated with each biliprotein in these phycobilisomes and the direction of energy transfer are also shown. The asterisk indicates that the bilin content given for allophycocyanin includes two bilins, which are each attached to a separate copy of an 18.5K polypeptide that forms a part of the core of this phycobilisome (*5, 6*).

examined the kinetics of emission from intact phycobilisomes with picosecond resolution. For these studies and for the measurements of individual phycobiliproteins the excitation source was the output of a tunable, picosecond, optical parametric source (OPS) (*11*) pumped by the third harmonic of a single amplified pulse from a passively mode-locked Nd:YAG laser system (YG400, Quantel). The OPS output typically consisted of 50- to 100-μJ, 10- to 15-psec pulses at a repetition rate of 10 Hz. The OPS wavelength could be continuously tuned from 450 to 630 nm. An ultrafast streak camera (IMACON 500, Hadland Photonics) with diode array readout (IDARSS, Tracor-Northern) was used to time-resolve the emission (*12*). The sample was excited with vertically polarized light. The emission was observed through a Glan-Taylor prism at 54.7° to minimize polarization effects. Data presented here typically represent the sum of the signal produced by 500 laser pulses. Such extensive averaging is necessary to achieve high signal-to-noise ratios at the low excitation energies required for these experiments. One measure of the overall efficiency and sensitivity of the detection system is that Raman scattering from the solvent can be routinely observed. Spectral filtering of the emission through 10-nm bandpass (full-width at half-maximum) interference filters allowed discrimination between the various components of the phycobilisome or phycobiliprotein emission. The broad tunability of the OPS was exploited to provide selective excitation at any point in any of the absorption bands of the chromophores of the samples examined, except the peak of the 650-nm band of allophycocyanin. Power densities used ranged from $<10^{12}$ to 10^{14} photons per square centimeter. For phycoerythrin, the biliprotein with the largest number of chromophores, the corresponding probabilities of exciting more than one chromophore per disk at these power density limits are <0.002 and 16.3 percent, respectively, for excitation at the peak absorption wavelength, 563 nm.

Synechocystis 6701 wild-type and CM25 mutant phycobilisomes were prepared from exponentially growing cyanobacterial cultures (*6*). Samples were stored in 0.75*M* sodium-potassium-phosphate buffer (*p*H 8.0)–0.75*M* sucrose at 4°C. Both steady-state emission spectra and time-resolved measurements indicated that the preparations were essentially free of dissociated material.

The wild-type and CM25 phycobilisomes differ (Fig. 1) in that the mutant lacks phycoerythrin disks at the ends of the rods whereas the wild-type particles contain an average of 1.6 disks per rod (*6*). The polypeptide compositions of the two phycobilisomes are identical with respect to all other components, as are their absorption and circular dichroism spectra above 600 nm. Electron micrographs of CM25 phycobilisomes show that they have shorter rods than wild-type particles, but otherwise their morphology is equivalent (*6*). Excitation of either phycobilisome at 570 nm results in virtually identical steady-state emission spectra with maxima at 676 nm. In wild-type phycobilisomes 570-nm light is absorbed by phycoerythrin (75 percent), phycocyanin (21 percent), and allophycocyanin (4 percent), whereas in CM25 phycobilisomes 83 percent of 570-nm light is absorbed by phycocyanin and virtually all of the remainder by allophycocyanin. The availability of both wild-type and mutant phycobilisomes offers a unique opportunity for comparative studies to determine the time required for energy absorbed by the phycoerythrin disks to be transferred to the phycocyanin disks.

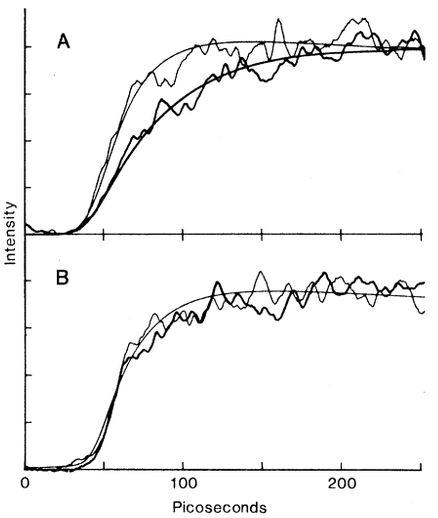

Fig. 2. Time-resolved 680-nm emission from terminal acceptors of *Synechocystis* 6701 (heavy trace) and CM25 (light trace) phycobilisomes. Excitation wavelengths were 570 nm (A) and 620 nm (B). The smooth curves are computer-generated fits to the data with rise times of 47 psec [*Synechocystis* 6701 (A)], 21 psec [CM25 (A)], and 25 psec (B).

The results of picosecond kinetics studies of these phycobilisomes are shown in Fig. 2. The data shown represent the accumulation of signal from 500 laser shots. The smooth curves are computer-generated fits to the data. Parameters for the fits were obtained through an iterative "convolute-and-compare" technique (*12*). Fig. 2A shows the time-resolved emission from the terminal energy acceptors of the phycobilisome at 680 nm after excitation at 570 nm. The best fits to the wild-type phycobilisome data give an average exponential rise time of 56 ± 8 psec. The CM25 data give an average best-fit exponential rise time of 25 ± 4 psec (*13*). The important result is that the difference in emission delay time between the two structures is 30 ± 5 psec (*14*). We take this time to be a direct measure of the average transit time for the energy from the outermost phycoerythrin disks to the closest phycocyanin disk in the phycobilisomes. Within experimental error, a decrease in the excitation power density by a factor of 10 did not change the observed time (*15*).

To ensure that the results for these two phycobilisomes can be compared, we performed an experiment for which the results should be independent of the structural differences between the two phycobilisomes. We tuned the excitation wavelength to the 620-nm absorption maximum of phycocyanin so that the phycocyanin complexes of the mutant and wild-type phycobilisomes were directly excited. The temporal profiles of 680-nm emission from the terminal acceptors on excitation at 620 nm are shown in Fig. 2B. As is readily apparent from the similarity of the profiles, there is no difference between the rates at which phycocyanin disks in the structures funnel quanta to the terminal acceptors. The average best-fit exponential rise time for wild-type phycobilisomes is 28 ± 4 psec and that for CM25 phycobilisomes is 25 ± 4 psec.

As a further independent measure of the transfer time between phycoerythrin and phycocyanin disks, emission from phycoerythrin in wild-type phycobilisomes was selectively excited at 530 nm and selectively detected at 570 nm. A typical time-resolved emission trace (Fig. 3) gives a best-fit decay constant of 27 psec. An important aspect of the curve in Fig. 3 relates to sample purity and reproducibility. The data in Fig. 3 were obtained from a 10-month-old sample. This sample showed a 2.5 percent contribution of long-lived emission arising from phycoerythrin that cannot undergo rapid energy transfer because it is no longer associated into a phycobilisome. In fresh (less than 1-week-old) samples, this long-lived emission was below the detection limit (≤0.1 percent). The presence of small amounts (≤2.5 percent) of dissociated phycobilisomes had no detectable effect on the rapidly decaying component of the phycoerythrin emission from intact particles. The average time constant for this decay, obtained from data taken on five differ-

ent preparations at two different power levels ($\sim 10^{12}$ and $\sim 10^{13}$ photons per square centimeter) over a period of 7 months, was 28 ± 4 psec.

All the independent measurements reported here are internally consistent. For example, if the observed times for transfer from phycocyanin to the terminal acceptor are considered, the measured phycoerythrin fall time predicts that overall transfer time from an intact wild-type phycobilisome should be 55 ± 10 psec, compared to the observed 56 ± 8 psec. Furthermore, if it is assumed that C-phycoerythrin in situ in *Synechocystis* 6701 phycobilisomes has a lifetime of ~1.5 nsec [the value found for the isolated biliprotein (*8*)] in the absence of energy transfer, the observed time constant indicates a 98.2 percent efficiency for transfer to phycocyanin. A similar estimate has been made (*10*) for the efficiency of transfer from the phycoerythrin of *Porphyridium cruentum* phycobilisomes.

From the comparative studies described above, we have determined the average time delay for energy transfer associated with the addition of an average of 1.6 phycoerythrin disks to otherwise unchanged rods. It is important to emphasize that the measured time delay is the sum of all time delays between the initial excitation of a phycoerythrin chromophore and the arrival of the excitation quantum at an adjacent phycocyanin disk, and to point out that the phycoerythrin disks themselves are complicated structures. For example, R-phycoerythrin carries 34-bilin chromophores in a $(\alpha\beta)_6\gamma$ disk-shaped assembly of 110 by 60 Å (*4*). This phycoerythrin hexamer represents an individual disk in the rod substructure. The bilin chromophores in a given biliprotein fall into two classes, sensitizing (s) and fluorescing (f) on the basis of the absorption and emission spectra of the disks (*7*). For example, there are several maxima in the absorption spectrum, as seen in Fig. 4 for R-phycoerythrin. The emission spectrum of R-phycoerythrin shows only a single peak at 578 nm. The two higher energy peaks in the absorption spectrum are assigned to s chromophores, while the lowest energy absorption peak corresponds to f chromophores. These assignments are supported by steady-state polarization measurements (*7*), which show that randomly polarized emission results from blue excitation, while substantial polarization is observed with red excitation. These observations provide evidence for energy flow between the s and f chromophores in the disks. As noted above for the overall energy transfer process, since this intradisk energy

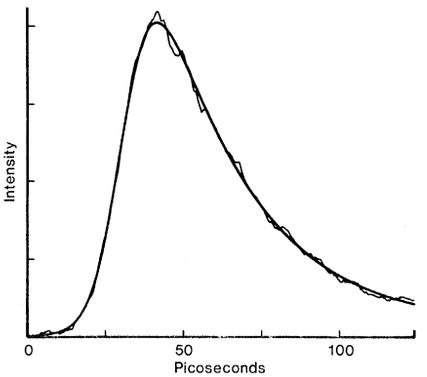

Fig. 3. Time-resolved emission from C-phycoerythrin in intact *Synechocystis* 6701 phycobilisomes for excitation at 530 nm and detection at 570 nm. The smooth curve is the best double-exponential fit to the data with fall times of 27 psec (97.5 percent amplitude) and 1.5 nsec (2.5 percent amplitude).

transfer competes efficiently with the fluorescence lifetime of the s chromophores, the internal s to f transfer time must be very short.

There are thus two distinct types of energy transfer events that can influence the time required for a quantum initially absorbed in a phycoerythrin disk to appear in a phycocyanin disk: intradisk s to f transfer and interdisk phycoerythrin to phycocyanin transfer. For phycobilisomes with more than one phycoerythrin disk per rod, interdisk phycoerythrin to phycoerythrin transfer must also be considered. To provide a foundation for understanding energy transfer in phycobilisomes, we examined the s to f energy transfer process in the individual phycobiliproteins *Gastroclonium coulteri* R-phycoerythrin and *Anabaena variabilis* allophycocyanin. The properties of R-phycoerythrin were noted above. Allophycocyanin carries 6 bilins per $(\alpha\beta)_3$ trimer and is a component of the core.

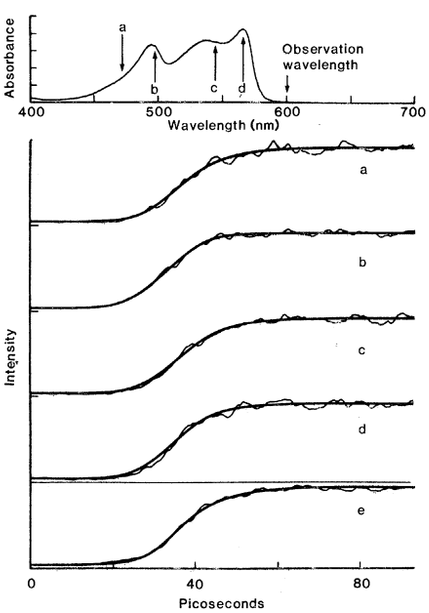

Fig. 4. Effect of excitation wavelength on emission rise time for isolated R-phycoerythrin. (Top) Excitation wavelengths shown in comparison to the R-phycoerythrin absorption spectrum. (Bottom) Rising portion of time-resolved, 600-nm emission from R-phycoerythrin for excitation at 470 nm (a), 496 nm (b), 540 nm (c), and 565 nm (d), and of 600-nm emission from rhodamine B in ethanol for excitation at 470 nm (e). Smooth curves show computer-generated fits to the data with rise times of 6.6 psec (a), 2.2 psec (b), 6.5 psec (c), 4.6 psec (d), and 7.0 psec (e).

Exposure of phycobilisomes to solutions of near neutral *p*H and moderate ionic strength (0.05 to 0.2*M*) leads to dissociation of the particles (*3*, *4*). Depending on the phycobilisome and the particular biliprotein, certain dissociation products of the phycobilisome retain the aggregation state and, either wholly or in large measure, the spectroscopic properties that they possessed in the native structure (*4*). R-phycoerythrin and allophycocyanin satisfy two important criteria. First, of the biliproteins studied to date, their aggregation state and spectroscopic properties are least altered by the isolation procedures. Second, they represent biliproteins that are at the two extremes of bilin content (*4*). R-phycoerythrin appears to fully retain the spectroscopic characteristics it exhibits in phycobilisomes. Allophycocyanin has a 650-nm absorbance ~20 percent lower and a 620-nm absorbance ~5 percent higher than that calculated for this trimer in the intact phycobilisome (*16*). All biliprotein preparations were obtained by established procedures (*17*) and were purified to homogeneity. All solutions used for spectroscopic measurements were monodisperse, as judged from absorption and emission characteristics.

By monitoring the effect of excitation wavelength on the temporal profile of emission from the f chromophores, the rate of energy transfer in a phycobiliprotein can be directly determined. Such data are shown in Fig. 4. For comparison, the emission profile of rhodamine B, known to have an essentially instantaneous rise time on this time scale (*18*), is also shown. Typical fits are superimposed on the data. Pertinent results are compiled in Table 1. Examination of these temporal profiles reveals that for R-phycoerythrin there is no delay in the rise of emission with blue excitation compared to the rise with red excitation. Similar results were found for allophycocyanin (Table 1). Our measurements put an upper limit of 8 psec (instrument-

limited) on the transfer from s to f chromophores. This finding is consistent with that of previous studies of isolated phycobiliproteins (*8*) and with the observation of a spectrally sharp onset of fluorescence at 565 nm in the steady-state spectrum and a lack of detectable emission at 550 nm that could be attributed to s chromophores.

This result has an important bearing on the interpretation of energy transfer dynamics in intact phycobilisomes. We have shown that communication of energy between s and f chromophores in individual disks is essentially instantaneous on the time scale of these experiments. Given the results for intact phycobilisomes, this indicates that disk-to-disk transfer must be the rate-limiting energy transfer process in these phycobilisomes. Together with the findings of previous structural analyses (*4–6*), these results show that, with respect to energy transfer, the lattice of ~625 light-harvesting chromophores in the *Synechocystis* 6701 wild-type phycobilisome (Fig. 1) functions as a linear five-point array.

If disk-to-disk transfer is the rate-limiting step, the kinetics of energy transfer in phycobilisomes can be modeled in a straightforward manner. In this model it is assumed that all disk-to-disk transfer times in rods are equivalent. Seemingly disparate results obtained by others on various intact phycobilisomes can be predicted solely on the basis of known phycobilisome structures. Gillbro *et al.* (*19*) recently compared the kinetics of energy transfer in the phycobilisomes of *Synechococcus* 6301 and a mutant, AN112, obtained from the laboratory of one of us (A.N.G.). The difference between the wild-type and mutant particles is limited to the rod substructures, which have an average of three phycocyanin disks for the wild type and one for the mutant (*20*). On excitation of these structures at 620 nm, recovery of absorption at 620 nm was 84 ± 8 psec for the wild type and 43 ± 8 psec for the mutant (*19*). The kinetic model predicts that the 41 ± 16 psec difference with a change in rod length of two disks arises from a disk-to-disk transfer time of 26 ± 8 psec, whereas a disk-to-disk transfer time of 24 ± 4 psec is predicted on the basis of the results presented here for *Synechocystis* 6701.

As reported above for wild-type *Synechocystis* 6701 phycobilisomes with an average of 1.6 phycoerythrin and 2 phycocyanin disks per rod, we find a rise time of 56 ± 8 psec for terminal acceptor emission at 680 nm on 570-nm excitation and a C-phycoerythrin fall time of 28 ± 4 psec. In a study of the hemidiscoidal phycobilisome *Rhodella violacea*, which is morphologically similar to *Synechocystis* 6701 but which has a different rod biliprotein composition, Holzwarth *et al.* (*21*) reported a rise time of 55 psec for 672-nm fluorescence on excitation at 571 nm, a B-phycoerythrin recovery time in a photobleaching experiment of 28 psec, and a B-phycoerythrin emission fall time of 34 psec. In studies of phycobilisomes from *Nostoc* species, which have a rod morphology nearly identical to that of *Synechocystis* 6701, Wong *et al.* (*8*) found a B-phycoerythrin fall time of 34 ± 13 psec. From these comparisons we feel that the qualitative features of the model proposed here are likely to hold for phycobilisomes of various morphologies and biliprotein compositions.

Table 1. Best-fit emission rise times for isolated phycobiliproteins and the rhodamine B control.

Excitation wavelength (nm)	Detection wavelength (nm)	Best-fit rise time (psec)
	R-phycoerythrin	
470	600	6.6*
496	600	2.2†
540	600	6.5‡
565	600	4.6§
	Allophycocyanin	
532	670	0.2
575	670	6.9
619	670	5.5
	Rhodamine B	
470	600	7.0‖
470	600	0.8
530	600	7.4
530	600	8.7
530	600	7.7

*Curve a in Fig. 4. †Curve b. ‡Curve c. §Curve d. ‖Curve e.

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13. The complex nature of the system under study implies that single or even multiple (*10*) exponential fits to the data represent a substantial oversimplification. Known structural heterogeneities for *Synechocystis* 6701 (Fig. 1) include single and double C-phycoerythrin disks in different rods and the fact that of the three core units, one is attached to four rods while each of the other two is attached to a single rod. The spectral separation between the various phycobiliproteins is good but not perfect. Single exponential fits to the data condense all these parallel decay channels into one. Even multiple exponential fits with excellent statistical validity will not necessarily yield accurate values for the true rate parameters. The approach taken in this study was to fit the data with appropriate single or multiple exponentials to obtain rates descriptive of the overall energy transfer process and to test the validity of such determinations with independent experiments and by comparison with previous results.
14. The uncertainty in the difference of these two values is smaller than the error in the minuend. The major source of error in either of the two separate measurements is inaccuracy in the determination of the true starting point of the emission. This source of error is removed in the relative measurement.
15. Previous research [F. Pellegrino, D. Wong, R. R. Alfano, B. A. Zilinskas, *Photochem. Photobiol.* **34**, 691 (1981)] showed that the observed fluorescence lifetimes in similar systems are strongly power-dependent. Extensive studies of the power dependence of the fluorescence kinetics were carried out to ensure that the present results are completely free of any artifacts due to excitation intensity. The present work was performed at excitation power densities up to 1000 times lower than the previously observed threshold for the onset of power-dependent effects.
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