FLUORESCENT TANDEM PHYCOBILIPROTEIN CONJUGATES

Emission Wavelength Shifting by Energy Transfer

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ABSTRACT A fluorescent tandem phycobiliprotein conjugate with a large Stokes shift was prepared by the covalent attachment of phycoerythrin to allophycocyanin. The efficiency of energy transfer from phycoerythrin to allophycocyanin in this disulfide-linked conjugate was 90%. A distinctive feature of this phycoerythrin-allophycocyanin conjugate is the wide separation between the intense absorption maximum of phycoerythrin ($\epsilon = 2.4 \times 10^6$ cm^{-1} M^{-1} at 545 nm) and the fluorescence emission maximum of allophycocyanin (660 nm). Energy transfer from a donor to a covalently attached acceptor can be used to adjust the magnitude of the Stokes shift. Tandem phycobiliprotein conjugates can be used to advantage in fluorescence-activated cell sorting, fluorescence microscopy, and fluorescence immunoassay analyses.

INTRODUCTION

Phycobiliproteins are a class of highly fluorescent proteins that form a part of the light-harvesting system in the photosynthetic apparatus of blue-green bacteria and of two groups of eukaryotic algae, red algae and the cryptomonads (1). Several properties of these proteins make them uniquely suited to function as components of specific fluorescent reagents for analyses of molecules and cells (2). They contain multiple bilin chromophores and hence have extremely high absorbance coefficients. For example, B-phycoerythrin contains 34 chromophores and has an ϵ_M of 2.4×10^6 cm^{-1} M^{-1} at 545 nm (3). Phycobiliproteins also have high fluorescence quantum yields (for B-phycoerythrin, $Q = 0.98$ [4]). Their strong visible absorption bands start at ~ 440 nm and extend into the far red. They are highly soluble and stable in aqueous solution and their fluorescence is not quenched by most biomolecules.

The magnitude of the Stokes shift is an important determinant of the ultimate sensitivity of a fluorescent analytical reagent. Rayleigh and Raman scattering interfere with the detection of low concentrations of an analyte, as does background fluorescence from other components. Hence, it is important to be able to manipulate the separation between the excitation and emission wavelengths. We show here that energy transfer from a donor to a covalently attached acceptor can be used to adjust the Stokes shift. Phycoerythrin is an especially suitable energy donor because of its broad and intense absorption between 450 and 570 nm. Allophycocyanin (λ_{max} 650 nm; ϵ_M 7.05×10^5 cm^{-1} M^{-1} per 100,000 daltons) was chosen as the energy acceptor because its emission maximum is at 660 nm, compared with 575 nm for phycoerythrin. Moreover, efficient energy transfer from phycoerythrin to covalently attached allophycocyanin was expected because of the favorable spectral overlap of this donor-acceptor pair. We report here the preparation and spectroscopic characteristics of this tandem phycobiliprotein conjugate.

METHODS

Preparation of B-Phycoerythrin-Allophycocyanin Conjugate

Reaction of B-Phycoerythrin with SPDP. To 1.0 ml (12.7 mg) of *Porphyridium cruentum* B-phycoerythrin (3) in 0.1 M Na phosphate, 0.1 M NaCl, pH 7.4, was added 10 μl (0.165 mg) *N*-succinimidyl 3-(2-pyridylthio)-propionate (SPDP) in anhydrous methanol. After 70 min, the reaction mixture was applied to a column of Sephadex G-25 (1.0 × 25 cm), equilibrated, and eluted with 0.1 M Na phosphate, 0.1 M NaCl, pH 7.4. The frontally eluted B-phycoerythrin-S-S-pyridyl derivative was stored at 4°C.

Preparation of Thiolated Allophycocyanin. To 0.38 ml (6 mg) of *Anabaena variabilis* allophycocyanin (5) in 0.1 M Na phosphate, 0.1 M NaCl, pH 7.4, was added 10 μl (0.169 mg) of SPDP in anhydrous methanol. After 60 min, the reaction was terminated by the addition of 20 μl 1 M dithiothreitol in the pH 7.4 buffer. After a further 30 min, the

reaction mixture was applied to a column of Sephadex G-25 (1.0 × 30 cm) and eluted with 0.1 M Na phosphate, 0.1 M NaCl, pH 7.4. Thiolated allophycocyanin was used immediately after gel filtration.

Spectroscopic Properties of the Modified Biliproteins. Both the absorption and fluorescence emission spectra of the B-phycoerythrin-S-S-pyridyl adduct and of thiolated allophycocyanin were the same as those of the unmodified proteins.

Conjugation Reaction. Thiolated allophycocyanin (2.52 mg, in 0.7 ml, 0.1 M Na phosphate, 0.1 M NaCl, pH 7.4) was mixed with the B-phycoerythrin-S-S-pyridyl derivative (5.12 mg, in 0.7 ml of the same buffer). After 18 h, the reaction mixture was dialyzed against 100 ml 0.1 M NaCl at room temperature for 180 min.

Purification of the B-Phycoerythrin–Allophycocyanin Conjugate. The dialyzed reaction mixture was applied to a column of hydroxylapatite (5-ml settled bed volume) equilibrated with 0.001 M Na phosphate, 0.1 M NaCl, pH 7.3. The column was washed with 20 ml of starting buffer, and then developed with 20 mM Na phosphate, 0.09 M NaCl, pH 7.3. This eluted a well-defined phycoerythrin zone. Elution with this buffer was continued until a barely detectable pink color remained in the eluate. At this point, the purple conjugate was eluted with 0.05 M NaCl, 0.1 M Na phosphate, pH 7.3. The molarity of B-phycoerythrin and of allophycocyanin in this column fraction was 4.25×10^{-6} M and 6.88×10^{-6} M, respectively, i.e., a molar ratio of allophycocyanin to phycoerythrin of 1.62. High-pressure liquid chromatography on a Waters instrument (Waters Associates, Millipore Corp,. Milford, MA) with a gel filtration column (G3000SW; Varian Associates, Inc., Palo Alto, CA) showed that column fractions containing the phycoerythrin-allophycocyanin conjugate were devoid of free phycoerythrin. The conjugate was used without further purification.

Spectroscopic Measurements. Absorption spectra were obtained on a spectrophotometer (model 25; Beckman Instruments, Inc., Fullerton, CA). Fluorescence spectra were obtained on a fluorimeter (model 44B; Perkin-Elmer Corp., Instrument Div., Norwalk, CT) equipped with a DCSU-2 corrected emission spectra unit, or on a Spex Fluorolog instrument (Spex Industries, Inc., Metuchen, NJ). The emission spectra reported here are nominally corrected spectra obtained with the fluorimeter (Perkin-Elmer Corp., Instrument Div.). Nanosecond emission kinetics were measured with a single-photon counting apparatus employing a mode-locked argon-ion laser (Spectra-Physics Inc., Mountain View, CA) with a synchronously pumped rhodamine 6G dye laser (Spectra-Physics Inc.) as the excitation source (6).

RESULTS

Absorption and Emission Properties of the Phycoerythrin-Allophycocyanin Conjugate

Phycoerythrin was covalently joined to allophycocyanin by reacting the activated -S-S-pyridyl derivative of phycoerythrin with thiolated allophycocyanin. The absorption and emission properties of this disulfide-linked conjugate are compared with those of the separate proteins in Fig. 1. The absorption spectrum of the conjugate is the sum of the contributions of its phycoerythrin and allophycocyanin components. The fluorescence emission spectrum of the conjugate excited at 500 nm showed a phycoerythrin contribution that peaked at 576 nm and an allophycocyanin contribution that peaked at 660 nm. The intensity of phycoerythrin fluorescence from the conjugate was 10% of that obtained from an equimolar concentration of

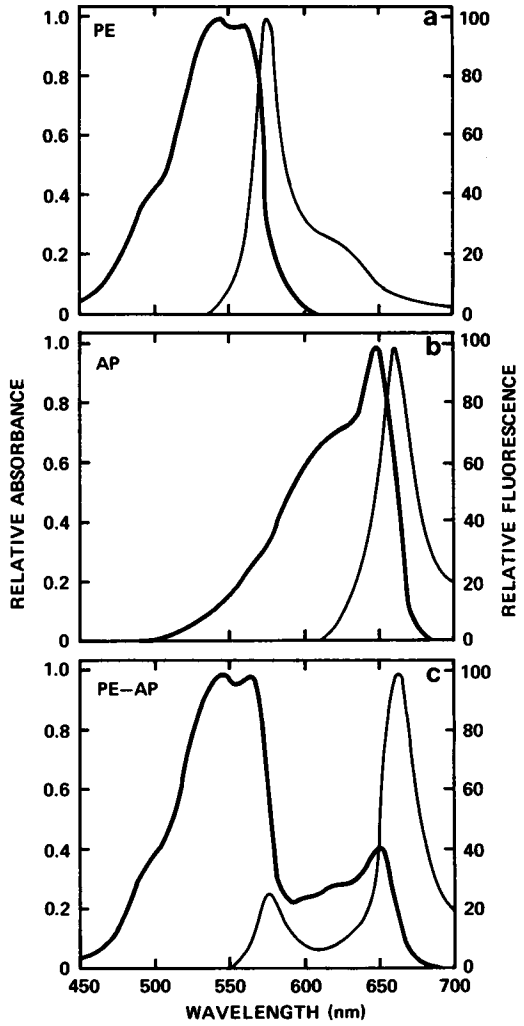

FIGURE 1 Absorption (—) and emission (—) spectra of (*a*) phycoerythrin (PE), (*b*) allophycocyanin (AP), and (*c*) the phycoerythrin-allophycocyanin conjugate (PE-AP). The emission spectra of PE and of PE-AP were excited at 500 nm, whereas that of AP was excited at 600 nm.

phycoerythrin alone. The excitation spectrum of the 660-nm fluorescence of the conjugate showed that the phycoerythrin fluorescence in the conjugate was quenched 90% because of highly efficient energy transfer to allophycocyanin. Only 5% of the 660-nm emission of the conjugate excited at 500 nm arose from direct excitation of the allophycocyanin component. Nearly all of the 660-nm emission of the conjugate was sensitized fluorescence. This is further documented in Fig. 2.

Reductive Cleavage of the Conjugate

The aliphatic disulfide bridge between phycoerythrin and allophycocyanin in the conjugate was stable in the absence of reducing agents. This disulfide bond can be cleaved by the addition of a high concentration of dithiothreitol (50 mM). The cleavage of the conjugate was monitored by the loss of energy transfer from phycoerythrin to allophycocyanin (Fig. 2). As the reduction proceeded, the 660-nm

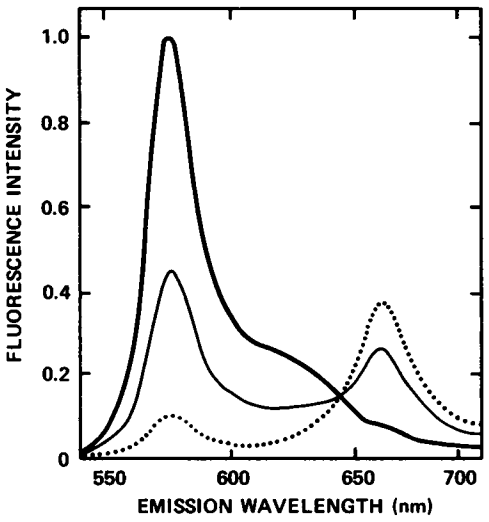

FIGURE 2 Cleavage of the phycoerythrin-allophycocyanin conjugate by dithiothreitol leads to an increase in the 576-nm phycoerythrin emission and a decrease in the 660-nm allophycocyanin emission. The excitation wavelength was 500 nm. The three emission spectra shown are: ---, before addition of 50 mM dithiothreitol; —, 8 min after addition; —, 2 h after addition.

emission of allophycocyanin decreased and the 576-nm emission of phycoerythrin increased. The initial rate of cleavage was ~1.5-fold faster than the final rate. The average rate of cleavage of the conjugate by 50 mM dithiothreitol was 9.8×10^{-4} s^{-1} at pH 7.5, 22°C. On reaching a plateau value in 2 h, the phycoerythrin emission intensity of dithiothreitol-treated conjugate was nearly the same as that of an equimolar solution of native phycoerythrin. This experiment also showed that the energy transfer observed with the phycoerythrin-allophycosyanin conjugate was a result of proximity brought about by covalent crosslinks rather than by noncovalent interactions. Nanosecond emission kinetics provided complementary information. The excited state lifetime of the phycoerythrin emission of the conjugate was <0.3 ns, compared with 2.1 ns for free phycoerythrin. There was no detectable 2.1-ns component in the emission of the conjugate. Following the addition of dithiothreitol, the amplitude of the τ = 2.1-ns component of the emission increased at the same rate as the increase in 576-nm fluorescence intensity.

DISCUSSION

This study shows that phycobiliprotein conjugates with novel fluorescence properties can be constructed. The aim here was to synthesize a fluorescent compound with a high absorption coefficient, a high fluorescence quantum yield, and a large Stokes shift. Phycoerythrin was chosen as the energy donor because it has an absorption coefficient of 2.4×10^6 cm^{-1} M^{-1} arising from 34 bilin chromophores. Allophycocyanin was used as the acceptor because its emission peak at 660 nm is far from the wavelength range over which the phycoerythrin donor has intense absorption (i.e., from ~470 to 560 nm). Highly efficient energy transfer to allophycocyanin was expected because of the high fluorescence quantum yield of phycoerythrin (Q = 0.98) and large magnitude of the spectral overlap integral ($J = 6.95 \times 10^{-13}$ cm^3 M^{-1}) in Förster's equation for dipole-dipole transfer (7, 8). This calculation of J is for transfer from phycoerythrin to a single allophycocyanin chromophore with an $\epsilon = 1.05 \times 10^5$ cm^{-1} M^{-1} at 650 nm. For n = 1.4 and K^2 = 0.67, the calculated R_0 distance for 50% transfer between a terminal phycoerythrin chromophore and a single allophycocyanin chromophore is 6.8 nm. The 34 bilin chromophores in phycoerythrin are distributed in a 240-kdaltons cylindrical protein with a diameter of 12 nm and a height of 6 nm. In allophycocyanin, the 6 bilin chromophores are distributed in a 100-kdaltons cylindrical protein with a diameter of 11 nm and a height of 3 nm. Some of the phycoerythrin chromophores in the conjugate may be separated from the nearest allophycocyanin chromophore by a distance considerably larger than R_0. For example, the transfer efficiency would be 5% for a phycoerythrin chromophore separated from an allophycocyanin chromophore by 110 Å, the center-to-center distance of the two protein disks placed edge-to-edge. Our observation of a 90% transfer efficiency indicated that the chromophores in phycoerythrin were coupled by resonance interactions so that excitation energy was very rapidly transferred within phycoerythrin to particular chromophores that were near the interface with allophycocyanin and hence transferred energy rapidly to this acceptor. Energy transfer between phycoerythrin and allophycocyanin in the conjugate was also enhanced by the presence of more than one allophycocyanin chromophore located within a distance of R_0 from the contact region with phycoerythrin.

The phycoerythrin-allophycocyanin conjugate is one of several tandem phycobiliprotein conjugates that can readily be synthesized. For example, phycoerythrin could be conjugated to phycocyanin (fluorescence λ_{max} = 648 nm) to give a fluorescent compound that has an emission spectrum between that of phycoerythrin and the phycoerythrin-allophycocyanin conjugate. A conjugate with an emission spectrum displaced further to the red could be constructed by linking phycoerythrin to allophycocyanin B [fluorescence λ_{max} = 680 nm (9)]. The wide separation between wavelengths of strong absorption and emission can be exploited in fluoresence immunoassays and other analyses to reject Rayleigh and Raman scattering. For example, Raman scattering from water at 3,100 cm^{-1} can be rejected by exciting the phycoerythrin-allophycocyanin conjugate at 500 nm and observing the emission at wavelengths longer than 660 nm, a separation of more than 4,800 cm^{-1}. Another attractive feature of tandem phycobiliprotein conjugates is that several compounds with different emission spectra can be simultaneously excited with high efficiency at a single excitation wavelength. This means that analyses for two or more components can be

simultaneously carried out with a single excitation beam. For example, it should be feasible to carry out multiparameter fluorescence-activated cell sorting using a series of tandem phycobiliprotein conjugates. Immunocytochemical localization of several antigenic markers could be performed by fluorescence microscopy employing these conjugates. The preservation of the favorable absorption and emission properties of phycobiliproteins following conjugation to each other or to other molecules indicates that they have broad applicability in fluorescence analyses of molecules and cells.

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