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EXCITATION SPECTRA FOR PHOTOSYSTEM I AND PHOTOSYSTEM II IN CHLOROPLASTS AND THE SPECTRAL CHARACTERISTICS OF THE DISTRIBUTION OF QUANTA BETWEEN THE TWO PHOTOSYSTEMS

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SUMMARY

The parameters listed in the title were determined within the context of a model for the photochemical apparatus of photosynthesis.

The fluorescence of variable yield at 750 nm at −196 °C is due to energy transfer from Photosystem II to Photosystem I. Fluorescence excitation spectra were measured at −196 °C at the minimum, F_0, level and the maximum, F_M, level of the emission at 750 nm. The difference spectrum, $F_M - F_0$, which represents the excitation spectrum for F_V is presented as a pure Photosystem II excitation spectrum. This spectrum shows a maximum at 677 nm, attributable to the antenna chlorophyll *a* of Photosystem II units, with a shoulder at 670 nm and a smaller maximum at 650 nm, presumably due to chlorophyll *a* and chlorophyll *b* of the light-harvesting chlorophyll complex.

Fluorescence at the F_0 level at 750 nm can be considered in two parts; one part due to the fraction of absorbed quanta, α, which excites Photosystem I more-or-less directly and another part due to energy transfer from Photosystem II to Photosystem I. The latter contribution can be estimated from the ratio of F_0/F_V measured at 692 nm and the extent of F_V at 750 nm. According to this procedure the excitation spectrum of Photosystem I at −196 °C was determined by subtracting 1/3 of the excitation spectrum of F_V at 750 nm from the excitation spectrum of F_0 at 750 nm. The spectrum shows a relatively sharp maximum at 681 nm due to the antenna chlorophyll *a* of Photosystem I units with probably some energy transfer from the light-harvesting chlorophyll complex.

The wavelength dependence of α was determined from fluorescence measurements at 692 and 750 nm at −196 °C. α is constant to within a few percent from 400 to 680 nm, the maximum deviation being at 515 nm where α shows a broad maximum increasing from 0.30 to 0.34. At wavelengths between 680 and 700 nm, α increases to unity as Photosystem I becomes the dominant absorber in the photochemical apparatus.

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INTRODUCTION

Previous studies of the fluorescence yield [1, 2] and energy transfer [3, 4] properties of chloroplasts led to a model [3, 4] for the photosynthetic apparatus in which yields of photochemistry, fluorescence and energy transfer could be expressed in terms of the fundamental photochemical parameters of the photosynthetic apparatus. The model assumes three major types of chlorophyll complexes: Photosystem I and Photosystem II units which contain antenna chlorophyll *a* and their respective reaction centers and a light-harvesting chlorophyll complex containing chlorophyll *a* and chlorophyll *b* which can transfer excitation energy to either of the two photosystem complexes. The three fluorescence emission bands which can be observed in fluorescence emission spectra from chloroplasts at −196 °C at 685, 695, and 735 nm were attributed to the three types of antenna chlorophyll *a* found, respectively, in the light-harvesting complex, in Photosystem II units and in Photosystem I units. At −196 °C chlorophyll *a* fluorescence at 730 nm or longer wavelengths is due to emission from Photosystem I units while that at 692 nm is due largely to emission from Photosystem II units [4].

The yields of fluorescence (quanta emitted/quanta absorbed by the photosynthetic apparatus) at 692 and 750 nm and the yield of energy transfer from Photosystem II to Photosystem I can be expressed as functions of the primary electron acceptors of Photosystem II present in the oxidized state, A.

$$\varphi_{F692} = \frac{\beta k_{F692}}{k_{F692}+k_{DII}+k_{T(II\to I)}+k_{TII}}\left(A+\frac{1-A}{1-\varphi_T\varphi_t}\right) \tag{1}$$

$$\varphi_{F750} = \frac{k_{F750}}{k_{F750}+k_{DI}+k_{TI}}\left(\alpha+\beta\varphi_{T(II\to I)}\right) \tag{2}$$

$$\varphi_{T(II\to I)} = \frac{k_{T(II\to I)}}{k_{F692}+k_{DII}+k_{T(II\to I)}+k_{TII}}\left(A+\frac{1-A}{1-\varphi_T\varphi_t}\right) \tag{3}$$

where α is the fraction of the absorbed quanta distributed to Photosystem I (either by direct absorption or by energy transfer from the light-harvesting complex); β is the fraction distributed to Photosystem II or dissipated in the light-harvesting complex ($\alpha+\beta = 1$), and the k's are the rate constants for the deexcitation of excited antenna chlorophyll *a* molecules in Photosystem II and Photosystem I by processes of fluorescence, k_F, nonradiative decay, k_D, transfer to the reaction center chlorophyll, k_T, and transfer from Photosystem II to Photosystem I, $k_{T(II\to I)}$. φ_T is the yield of energy transfer from the antenna chlorophyll to the reaction center chlorophyll of Photosystem II ($\varphi_T = k_{TII}/\Sigma k_{II}$). If excitation energy in Photosystem II is trapped by an open reaction center, photochemistry, k_p, is accomplished and no increase of fluorescence occurs; if the energy is trapped by the reaction center chlorophyll of a closed Photosystem II reaction center (the 1−A fraction) that energy may be transferred back to the antenna chlorophyll, k_t, or dissipated at the reaction center chlorophyll in a nonradiative decay process, k_d. The yield of energy transfer from the closed Photosystem II reaction centers back to the antenna chlorophyll, φ_t, is $k_t(k_t+k_d)^{-1}$.

It is apparent from the equations that the yield of energy transfer from Photo-

system II to Photosystem I should have a constant part, $\varphi_{T(II\to I)(0)}$, and a variable part, $\varphi_{T(II\to I)(V)}$, that are in the same ratio as the constant and variable parts of φ_{F692}. It is also apparent from the equations that all fluorescence of variable yield, including that at 750 nm, is controlled by the state of the reaction centers of Photosystem II (no fluorescence yield changes are related to the primary photochemistry of Photosystem I [3, 4]); the fluorescence of variable yield which can be observed at 750 nm at −196 °C is ascribed to energy transfer from Photosystem II to Photosystem I.

It was shown previously that relative values of α could be determined from fluorescence induction curves measured at −196 °C at 690 and 730 nm. In the present work we will make such measurements at 692 and 750 nm to determine how α depends on the wavelength of excitation. We will also examine fluorescence excitation spectra at 750 nm at −196 °C measured at the minimal F_0 level and the maximal F_M level. The difference spectrum between these two excitation spectra ($F_M - F_0$) represents the excitation spectrum of F_V at 750 nm and as such should represent a pure Photosystem II excitation spectrum. Given the pure Photosystem II excitation spectrum we should be able to subtract the $\beta\varphi_{T(II\to I)(0)}$ contribution from the excitation spectrum of F_0 (see Eqn 2) and obtain a pure excitation spectrum of Photosystem I, i.e. that part of F_0 at 750 nm which is due to α.

MATERIALS AND METHODS

Chloroplasts were prepared by homogenizing 50 g of commercial spinach leaves in a Waring Blender for 15 s in 250 ml of a solution containing 0.4 M sucrose, 10 mM NaCl, 5 mM $MgSO_4$ and 5 mM Tris-HCl (pH 7.8). The homogenate was squeezed through fine mesh cloth and centrifuged for 7 min at $1000 \times g$. The pellet was suspended in 20 ml of the above solution and heavy particles were removed by centrifugation at $300 \times g$ for 1 min. The supernatant was then centrifuged at $1000 \times g$ for 7 min and the pellet was resuspended in 5 ml of the same suspending solution to give a chlorophyll concentration of about 3 mg/ml and kept near 0 °C. Prior to measurement, 10 μl of this concentrated chloroplast suspension was diluted with a reaction medium (20 mM NaCl, 5 mM $MgCl_2$ and 15 mM Tris-HCl, pH 7.8) to give a final chlorophyll concentration indicated in the figure legends. The samples, frozen to −196 °C in a vertical, cylindrical cuvette, had a thickness of about 1 mm.

Fluorescence was measured at −196 °C from the bottom of the sample with the exciting light incident on the top similar to methods reported previously [4]. Fluorescence was measured at 692 nm through a set of filters (two 690 nm interference filters, a Corning 9830 filter and a Toshiba VR 65 filter) which gave a 7 nm passband between the 50 % transmission wavelengths and at 750 nm (750 nm interference filter with Corning 9830 and 2600 filters) with a 10 nm passband. The minimal, F_0, and maximal, F_M, levels of fluorescence were measured at 692 and 750 nm with monochromatic excitation from a Cary Model 14 monochromator with the slits set at 2 to 3 mm depending on the wavelength. The intensity of fluorescence was measured before and after a saturating irradiation with blue light. In one experiment the fluorescence excited with 633 nm light from a small He-Ne laser (50 μW/cm^2) was measured as a function of time simultaneously at 692 and 750 nm from a single sample by means of a half-silvered mirror and two phototubes connected to the two axes of an X-Y recorder.

Fluorescence excitation spectra were recorded with our computer-linked, single-beam spectrophotometer [5] by measuring the intensity of fluorescence at 692 or 750 nm excited by the monochromatic excitation beam as the monochromator scans the spectrum. The current from the phototube was measured in either a linear mode (as in Fig. 2) or a logarithmic mode (as in Fig. 4). The intensity of the monochromatic beam in these experiments was sufficiently low that the F_0 level of fluorescence at −196 °C was not altered significantly by the measurement. The intensity distribution of the monochromatic exciting light was measured so the excitation spectra could be corrected for the quantum flux of the exciting light. The quantum flux was constant within ±10 % in the spectral region between 600 and 700 nm so that no correction was applied to excitation spectra measured over this region. The correction was applied to spectra measured over the larger region of 400 to 650 nm. Difference spectra were obtained by subtracting two excitation spectra with the computer. All spectra were plotted directly from the computer to an X-Y recorder.

RESULTS AND DISCUSSION

The case was made previously [3, 4] that the photochemistry of Photosystem I at −196 °C does not cause any fluorescence yield changes. The fluorescence of variable yield which can be measured in the long wavelength region due to Photosystem I fluorescence is attributed to energy transfer from Photosystem II to Photosystem I. According to that view and to Eqns 1, 2 and 3, the state of the Photosystem II reaction centers should control F_V at 692 and 750 nm to exactly the same extent. That proposition was tested at −196 °C by measuring fluorescence induction changes at 750 nm and 692 nm simultaneously from a single sample on the two axes of an X-Y recorder. The straight line plot (Fig. 1) as the fluorescence increases from F_0 to F_M demonstrates that the kinetics of the fluorescence yield changes are identical at both wavelengths. Such straight line plots at −196 °C were obtained for any pair of emission wavelengths tested between 680 and 770 nm. It is worth noting that, according to Eqn 2, the extrapolation of the straight line in Fig. 1 back to the X axis gives a relative value α.

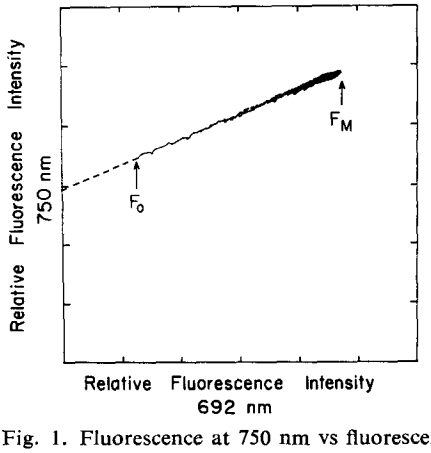

Fig. 1. Fluorescence at 750 nm vs fluorescence at 692 nm measured from a sample of chloroplast (50 μg chlorophyll/ml) at −196 °C as a function of time as the fluorescence yield increases from the F_0 level to the F_M level due to irradiation (50 μW/cm^2) at 633 nm.

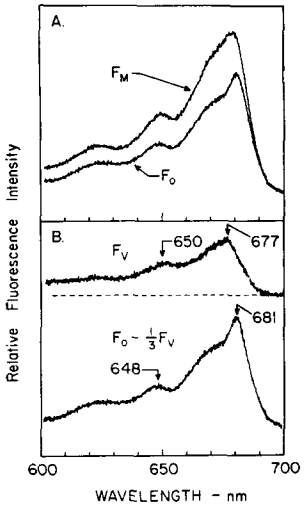

Fig. 2. (A) Excitation spectra for fluorescence at 750 nm from chloroplasts (50 μg chlorophyll/ml) at -196 °C measured at the F_0 level and the F_M level. (B) F_V is the difference between the excitation spectra in part A, $F_M - F_0$. The dashed line is the zero line for that difference spectrum. $F_0 - 1/3\ F_V$ is the spectrum for F_0 minus 1/3 the spectrum of F_V.

Excitation spectrum of Photosystem II

Once it is recognized that F_V at 750 nm is excited solely by Photosystem II, it becomes a relatively straightforward project to determine the excitation spectra of Photosystem II and Photosystem I. Excitation spectra for fluorescence at 750 nm were measured at -196 °C at the minimal F_0 level and the maximal F_M level. It is apparent in Fig. 2 that the main excitation band for both of these spectra is at 680 to 681 nm. The difference spectrum between these two spectra is the excitation spectrum for F_V at 750 nm. The excitation spectrum for F_V shows bands at 677 and 650 nm and a shoulder at 670 nm, but no indication of an excitation band at 681 nm. We attribute the 681 nm band to antenna chlorophyll *a* in Photosystem I, the 677 nm band to antenna chlorophyll *a* in Photosystem II and the 670 nm shoulder and 650 nm maximum to the chlorophyll *a* and *b* of the light-harvesting complex. We take the excitation spectrum of F_V to be a pure Photosystem II excitation spectrum (encompassing direct excitation of Photosystem II and energy transfer from the light-harvesting complex).

Excitation spectrum of Photosystem I

Eqn 2 indicates that F_0 at 750 nm is comprised of two parts; α, due to the fraction of absorbed quanta which excites Photosystem I directly, either by direct absorption or by energy transfer from the light-harvesting complex, and $\beta\varphi_{T(II\to I)(0)}$ due to energy transfer from Photosystem II. As has been noted previously [3, 4], $\varphi_{T(II\to I)}$. should have a constant part and a variable part that are in the same ratio as the constant and variable parts of φ_{F692}. Thus,

$$\beta\varphi_{T(II\to I)(0)} = \frac{\varphi_{F692(0)}}{\varphi_{F692(V)}}\,\beta\varphi_{T(II\to I)(V)} \tag{4}$$

where $\beta\varphi_{1(II\to I)(V)}$ is the extent of F_V at 750 nm. In the chloroplasts used in these experiments, $\varphi_{F692(0)}$ is approximately 1/3 $\varphi_{F692(V)}$. Thus if we subtract 1/3 of the excitation spectrum of F_V at 750 nm from the excitation of F_0 at 750 nm we should obtain the excitation spectrum for the α part of the excitation. This difference spectrum ($F_0 = 1/3\ F_V$) in Fig. 2 shows the main excitation band at 681 nm due to the chlorophyll *a* in Photosystem I, a broad shoulder at 670 nm which can be attributed to the chlorophyll *a* in the light-harvesting complex and a band at 648 nm which is probably due at least in part to the chlorophyll *b* in the light-harvesting complex. However, the position of this latter band at 648 instead of 650 nm raises some question as to whether the band is due entirely to chlorophyll *b*. It is possible that the 681 nm absorbing form of chlorophyll has a small satellite band in the 645 nm region. We conclude, however, that the pure Photosystem I excitation spectrum (due to α) includes some energy transfer from the light-harvesting complex.

The subtraction of 1/3 F_V from F_0 at 750 nm represents an upper limit for such a correction term. In a previous discussion of energy transfer [4] the model was refined to include two types of energy transfer; an energy transfer from the antenna chlorophyll of Photosystem II to Photosystem I, the $k_{T(II\to I)}$ process, and a more-or-less direct energy transfer from the reaction center chlorophyll of Photosystem II to Photosystem I, a $k_{t(II\to I)}$ process. If that version of the model were used here, somewhat less F_V would be subtracted from F_0. However, the general character of the excitation spectrum for the α component of the 750 nm fluorescence would be essentially the same.

Spectral characteristics of α

The excitation spectrum for Photosystem I includes α, the yield of fluorescence of Photosystem I, which we assume is a constant, and the absorption characteristics of the photosynthetic apparatus. In theory we should be able to obtain the spectral characteristics of α by dividing the excitation spectrum for Photosystem I by the absorption spectrum of the photosynthetic apparatus. However, we do not know this absorption spectrum with adequate precision because accessory pigments (such as carotenes) may not transfer excitation energy to the photosynthetic apparatus with 100 % efficiency. Since $\alpha+\beta = 1$ by definition, those accessory pigment molecules which do not transfer excitation energy to one of the chlorophyll complexes are not considered as part of the photosynthetic apparatus. Nevertheless, the wavelength dependency of α can be determined independent of the absorption characteristics. We showed previously [4] that relative values of α can be determined from fluorescence measurements.

$$\frac{\alpha}{F_{0750}} = 1 - \frac{(F_V/F_0)_{750}}{(F_V/F_0)_{692}} = 1 - \frac{(F_M/F_0)_{750} - 1}{(F_M/F_0)_{692} - 1} \tag{5}$$

Fluorescence was measured at −196 °C for emission at 692 and 750 nm with different wavelengths of exciting light. The minimal F_0 and maximal F_M levels were determined from these fluorescence measurements and the ratios of F_M/F_0 at 692 and 750 nm are plotted in Fig. 3 as a function of the wavelength of excitation. Our model predicts that F_M/F_0 at 692 nm should be a constant equal to $(1-\varphi_T\varphi_t)^{-1}$ [2, 3]: the measurements of F_M/F_0 at 692 nm confirm that the ratio is constant (within experimental limits) over the range of excitation from 400 to 650 nm (measurements of

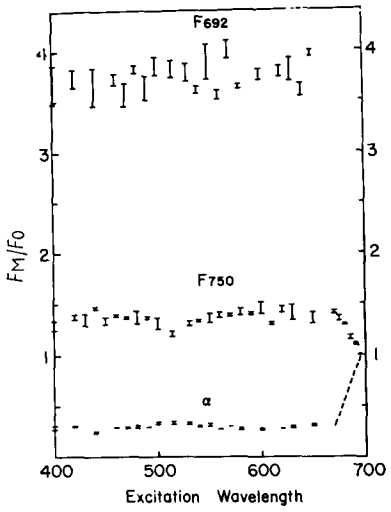

Fig. 3. Ratios of F_M/F_0 measured at 692 and 750 nm as a function of the excitation wavelength from chloroplasts (15 μg chlorophyll/ml) at -196 °C. α is plotted on the same scale taking the absolute value to be 0.30 in the blue.

692 nm fluorescence could not be made at longer wavelengths of excitation because of the need to color separate fluorescence from excitation). The fluorescence of 750 nm is a function of both α and β so that the ratio of F_M/F_0 at 750 nm will vary with wavelength if α and β vary with wavelength. However, the data in Fig. 3 show that F_M/F_0 at 750 nm is largely independent of wavelength between 400 and 680 nm (with the possible exception of a small minimum at 515 nm) so that the values of α should be essentially constant over this wavelength range. (In contrast to spinach chloroplasts, measurements of α in blue-green algae (not reported here) show marked changes across the visible spectrum.) From our previous study of fluorescence in the presence and absence of Mg^{2+} [4] we were able to calculate the absolute value of α to be about 0.30 with broad band blue excitation. We assume that same value here in the blue region to establish an absolute scale for α. At excitation wavelengths between 680 and 700 nm the ratio F_M/F_0 at 750 nm decreases to unity, because of the decline of Photosystem II excitation, and α increases to unity. The constancy of α throughout the major part of the visible spectrum probably reflects the dominant absorbance of the light-harvesting complex throughout this region. Any absorbance differences between the Photosystem I and Photosystem II units, which would confer a wavelength dependency to α, would be minimized by the larger absorbance of the light-harvesting complex up to 680 nm; at longer wavelengths Photosystem I becomes the dominant absorber and α increases toward unity.

The determination of α in Fig. 3 was subject to experimental uncertainties in the measurement of F_M/F_0 of approximately 10%. Measurements of excitation spectra can be made with much greater precision especially at the F_M level where we need not be concerned with any actinic effect of the excitation light. The ratio of the fluorescence, F_{750}/F_{692}, measured at the F_M level with the same excitation light can be expressed as:

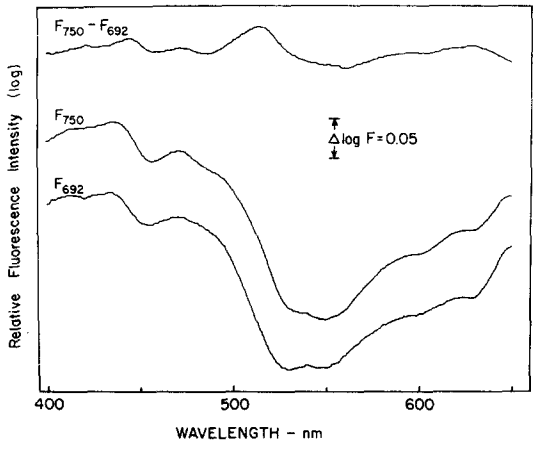

Fig. 4. Excitation spectra at the F_M level measured at 692 and 750 nm from chloroplasts (6 μg chlorophyll/ml) at -196 °C and the difference spectrum between the two excitation spectra.

$$R = \frac{F_{M750}}{F_{M692}} = \frac{\alpha+\beta\varphi_{T(II\to I)(M)}}{\beta} K \tag{6}$$

where K is a constant which incorporates the fluorescence yields at 692 and 750 nm as well as the difference in the sensitivity of the phototube at the two wavelengths. $\varphi_{T(II\to I)(M)}$ is a constant so that if α and β vary with wavelength, R should vary with wavelength; or conversely, if α is a constant as the data of Fig. 3 indicate, β will be constant ($\alpha+\beta = 1$) and R should be independent of wavelength. Fig. 4 shows excitation spectra from 400 to 650 nm corrected for equal incident quantum flux for fluorescence at 692 and 750 nm measured at the F_M level from the same sample frozen to -196 °C. The excitation spectra were measured on a logarithmic scale so the difference spectrum, $\log F_{M750} - \log F_{M692}$, would be equivalent to the spectrum of $\log R$. The difference spectrum is fairly flat with a few broad maxima. The major peak at 515 nm represents an increase of $\log R$ of about 0.05. If we differentiate $\log R$ with respect to α we obtain:

$$\frac{d(\log R)}{d\alpha} = \frac{0.434}{\beta(\alpha+\beta\varphi_{T(II\to I)(M)})} \tag{7}$$

Taking the value of α to be 0.30 and the value of $\varphi_{T(II\to I)(M)}$ to be 0.25 from previous results [4], $d(\log R)/d\alpha = 1.3$. Thus, the maximum difference of $\log R$ of 0.05 corresponds to a $\Delta\alpha$ of 0.038 or a $\Delta\alpha/\alpha$ of about 13 %. The maximum in the difference spectrum at 515 nm indicates an increase of α from 0.30 to 0.34. This small increase of α is corroborated by the slight increase in the ratio of F_M/F_0 at 750 nm shown in Fig. 3 for excitation at 515 nm but these measurements do not have adequate precision to reveal such small changes of α with certainty: within the context of the model, the difference spectrum reveals changes of α of about 1 %. We interpret the maximum in the difference spectrum at 515 nm to indicate a carotenoid pigment which shows some degree of preferential energy transfer to Photosystem I.

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