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Resolution of component spectra for spinach chloroplasts and green algae by means of factor analysis

Michael A. Marchiarullo * and Robert T. Ross **

Department of Biochemistry, Ohio State University, 484 West 12th Avenue, Columbus, OH 43210 (U.S.A.)

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Using a multivariate analysis method known as factor analysis, we have studied two distinct components in the room temperature fluorescence of broken spinach chloroplasts and of two species of algae. This method provides excitation and emission spectra of the components without physical separation and with a minimum of assumptions about the shape of the spectra. In spinach chloroplasts, both components had excitation maxima at 680 nm. Component 1, containing mostly Photosystem I, had a stronger far-red absorbance, and had an emission maximum at 683 nm with a very pronounced shoulder from 710 to 750 nm. Component 2, containing mostly Photosystem II, had a slightly stronger absorbance at 650 nm. Its emission maximum was at 682 nm with a weak band from 710 to 750 nm, similar to those of isolated Photosystem II particles. The spectra from *Chlorella pyrenoidosa* and *Scenedesmus quadricauda* were different from those of spinach. Component 1 in algae had an excitation maximum of 680 nm and an emission maximum of 690 nm, without the strong 710–750 nm shoulder. Algal component 2 had peak excitation at 670 nm and peak emission at 685 nm. In both spinach chloroplasts and the algae, the integrated fluorescence from component 1 was 20–40% of the total. The Stepanov temperature of component 2 was within 10 K of the ambient temperature, providing strong evidence for good equilibration within and between the excited states of Photosystem II. The Stepanov temperature of component 1 was less well defined, with a median value of 330 K or more, suggesting a lack of equilibration in Photosystem I. Thus, calculations of free-energy yield based on an assumption of excited-state equilibrium should be valid for Photosystem II, but may not be valid for Photosystem I.

Introduction

Several different chlorophyll-protein complexes have been isolated, falling into one of three main categories [1–3]: those responsible for the photochemical reactions of Photosystem I (PS I), those responsible for the photochemical reactions of Photosystem II (PS II) and those with no intrinsic photochemical activity but serving as a light-gathering system (LHC). Within these broad categories, there is additional complexity, such as two distinct types of PS II [4,5], and a detachable antenna subunit in PS I [6,7].

Energy transfer occurs among the several kinds of complexes. The principal LHC complex passes excitations to PS II [8–11], increasing the antenna size of this photosystem. Transfer can occur be-

* Present address: Pharmaceutics Department, F-70, Smith, Kline and French Laboratories, Philadelphia, PA 19101, U.S.A.
** To whom correspondence should be addressed.
Abbreviations: LHC, light-harvesting chlorophyll protein; PS, Photosystem.

tween PS II complexes, allowing photon energy absorbed by a PS II unit with a closed trap to be passed to one with an open trap. And, there may be some 'spill-over' of excitations from PS II to PS I [10–11].

Excitation transfer raises the question of how many spectrally and kinetically independent pigment components are present in an intact photosynthetic system. Chromatography might indicate the presence of many chemically distinct pigment-protein complexes, and derivative spectroscopy [12–14] or spectral curve resolution [15–16] might indicate the presence of many different electronic transitions, but these are not all spectrally and kinetically independent because of rapid excitation transfer.

The first and still the best evidence of at least two independent pigment components in oxygen-evolving photosynthesis is the existence of different action spectra for PS I and PS II. Matrix rank analysis, first applied to fluorescence spectra by Weber [17], was used to show the presence of at least two components in the room-temperature fluorescence spectra of spinach chloroplasts [18] and in the green alga *Chlorella* [19].

Factor analysis is an extension of matrix rank analysis which, under favorable conditions, permits the determination not only of the number of components, but also of the spectra and other properties of these components. Factor analysis has been used to resolve possible absorption spectra of dyes present in different concentrations for a series of solutions [20,21], and to resolve the excitation and emission spectra of binary dye mixtures [22].

This method has a major advantage over other decomposition methods, such as gaussian analysis, in that the number of components used, and the spectra deduced, are independent of assumptions about band shape.

We report here the application of factor analysis to the fluorescence of broken chloroplasts and intact green algae, with the goal of obtaining the spectra and other properties associated with the components enumerated by matrix analysis.

Theory

This section describes the general theory of spectroscopic factor analysis [22–23], using a mixture of two fluorescing compounds with distinct excitation and emission spectra as an example. Our particular application of this technique is described in the Materials and Methods section.

For such a mixture, excitation at different wavelengths will give different emission spectra. For excitation at wavelength i with emission measured at wavelength j, the intensity of measured emission may be written as:

$$y_{ij} = e_{i1}f_{1j} + e_{i2}f_{2j} \tag{1}$$

where e_{i1} and e_{i2} are the relative rates at which illumination at wavelength i excites fluorescence from components 1 and 2, and f_{1j} and f_{2j} are the relative emission spectra of components 1 and 2 at wavelength j. The relative concentration, absorbance and fluorescence quantum yield of each component is included within e_{i1} and e_{i2}.

If fluorescence is measured with m different excitation wavelengths and n different emission wavelengths, then the results can be written in matrix notation as:

$$\begin{pmatrix} y_{11} & \cdots & y_{1n} \\ y_{21} & \cdots & y_{2n} \\ \cdot & \cdots & \cdot \\ \cdot & \cdots & \cdot \\ \cdot & \cdots & \cdot \\ y_{m1} & \cdots & y_{mn} \end{pmatrix} = \begin{pmatrix} e_{11} & e_{12} \\ e_{21} & e_{22} \\ \cdot & \cdot \\ \cdot & \cdot \\ \cdot & \cdot \\ e_{m1} & e_{m2} \end{pmatrix} \begin{pmatrix} f_{11} & \cdots & f_{1n} \\ f_{21} & \cdots & f_{2n} \end{pmatrix} \tag{2}$$

This can be more simply written as:

$$\mathbf{Y} = \mathbf{EF} \tag{3}$$

The rows of **F** are the fluorescence spectra of the two components, while the columns of **E** are the corresponding excitation spectra.

The goal of factor analysis is to start with **Y** and work backwards to find **E** and **F**.

The first step is to perform an operation known as a singular value decomposition [24–26] on the data matrix **Y**, which may be accomplished using a standard library program (such as subroutine LSVDF, International Mathematical and Statistical Libraries, Houston, TX, U.S.A.). This decomposition of a rectangular matrix is somewhat equivalent to finding the eigenvectors and eigenvalues of a square matrix. In factor analysis it replaces the algebraically equivalent but computa-

tionally inferior calculation of eigenvalues and -vectors of covariance matrixes constructed from the data matrix.

Singular value decomposition expresses the original data matrix, **Y**, as the sum of vector and scalar products:

$$\mathbf{Y} = \sum_{i=1}^{n} A_i S_i B_i \tag{4}$$

where A_i and B_i are row and column vectors, respectively, and S_i is a scalar called the singular value. n is the number of singular value-vector pair sets needed to reproduce **Y**.

The largest singular value, together with its associated vector pair, is the best least squares fit to **Y** for $n = 1$. A new matrix, $\mathbf{R}_1$, can be defined:

$$\mathbf{R}_1 = \mathbf{Y} - A_1 S_1 B_1 \tag{5}$$

The second singular value-vector pair combination is the best least squares fit to $\mathbf{R}_1$. A new residual matrix, $\mathbf{R}_2$, can be defined in a similar manner:

$$\mathbf{R}_2 = \mathbf{R}_1 - A_2 S_2 B_2 \tag{6}$$

Each additional term $A_i S_i B_i$ is the best least squares fit to $\mathbf{R}_{i-1}$. The process is repeated until the matrix is reproduced exactly.

In matrix notation. **Y** can be represented as:

$$\mathbf{Y} = \mathbf{ASB} \tag{7}$$

where **A** is an m by m matrix, **B** is an n by n matrix, and **S** is an m by n diagonal matrix. The diagonal elements of **S** are positive or zero, and are sorted in order of size, the largest first. The kth row of **A** and kth column of **B** represent the pair of vectors associated with the kth singular value.

If there were no errors in the data, **Y** would have the same number of non-zero singular values as there are components in the sample measured. With real data containing experimental noise, all of the singular values will be non-zero. In our example, the two largest singular values, and their associated vectors in **A** and **B**, represent true signal plus some noise. Only these first two vector pairs are used in attempts to resolve component spectra, since they contain all the information in the data about the behavior of the fluorescing compounds. The remaining singular values and vectors represent pure noise, and are not used, since they would only make resolution of the component spectra more difficult.

In the absence of prior knowledge of the number of components, there are several methods available to determine how many of the singular values and vectors represent true components [22–24,27]. Our procedure is described in the next section.

For our two-component example, we retain only the two largest singular values and their associated vectors in **A** and **B**. We can now define two new matrices: **E′**, an m by 2 matrix, containing the first two rows of **A** multiplied by their corresponding singular value; and F′, a 2 by n matrix, containing the first two columns of **B**. Thus we have:

$$\mathbf{Y}' = \mathbf{E}'\mathbf{F}' \tag{8}$$

where **Y′** is the data matrix with most noise removed. With ideal, noise-free data, **Y′** and **Y** would be identical.

Note that the dimensions of **E′** and **F′** correspond to those of **E** and **F**, and that Eqns. 3 and 8 suggest a close relationship between **E**, **F** and **E′**, **F′**. However, the vectors within **E′** or **F′** are mutually orthogonal and are not themselves the spectra which comprise **E** and **F**.

The problem now is to turn the abstract matrices **E′** and **F′** into the corresponding matrices **E** and **F**, which have real spectroscopic meaning. The two excitation spectra, the columns of **E**, are linear combinations of the two singular vectors which are the columns of **E′**. To accomplish this combination, we define a 2 by 2 transformation matrix **T** so that:

$$\mathbf{E} = \mathbf{E}'\mathbf{T} \tag{9}$$

Similarly, the two emission spectra, the rows of **F**, are linear combinations of the two singular vectors which are the rows of **F′**. Comparing Eqns. 3, 8 and 9, we note that the matrix transforming **F′** into **F** must be the inverse of **T**:

$$\mathbf{F} = \mathbf{T}^{-1}\mathbf{F}' \tag{10}$$

so that:

$$\mathbf{Y} = \mathbf{E'F'} = \mathbf{E'TT^{-1}F'} = \mathbf{EF}$$

Going greater mathematical detail for our two-component system, the matrices $\mathbf{E'}$, $\mathbf{F'}$, $\mathbf{E}$ and $\mathbf{F}$ can be thought to consist of vectors as follows:

$$\mathbf{E'} = \begin{pmatrix} A_1 \\ A_2 \end{pmatrix}$$

$$\mathbf{F'} = (B_1 \; B_2)$$

$$\mathbf{E} = \begin{pmatrix} E_1 \\ E_2 \end{pmatrix}$$

$$\mathbf{F} = (F_1 \; F_2)$$

If the transformation matrix $\mathbf{T}$ is described as:

$$\mathbf{T} = \begin{pmatrix} \alpha & \beta \\ \gamma & \delta \end{pmatrix}$$

then its inverse is:

$$\mathbf{T^{-1}} = \frac{\begin{pmatrix} \delta & -\beta \\ -\gamma & \alpha \end{pmatrix}}{\alpha\delta - \beta\gamma}$$

and:

$$E_1 = \alpha A_1 + \gamma A_2$$

$$E_2 = \beta A_1 + \delta A_2$$

$$F_1 = \frac{+\delta B_1 - \beta B_2}{\alpha\delta - \beta\gamma}$$

$$F_2 = \frac{-\gamma B_1 + \alpha B_2}{\alpha\delta - \beta\gamma}$$

Identification of the appropriate transformation matrix can be carried out in two ways. In the first, target testing [23], a direct vector-matrix multiplication is used to find the transformation matrix which gives a least squares best fit between the spectra from factor analysis and the spectra of chromophores known or suspected of being present in the sample. This requires detailed prior knowledge of the sample of interest, but can yield unambiguous results.

In the second approach, general features known to be present in the spectra are used to limit the choice of transformation matrix. This method requires less detailed information, but it may not yield unique spectra as solutions [20–23]. In the simplest application of this approach to excitation-emission spectra, one simply requires that neither excitation nor emission spectra can have negative values at any point. This requirement will produce bands of possible spectra, with exact spectra resolved only if there are wavelengths at which one component is the sole contributor to the measured spectrum [20–23]. Warner et al. [22] describe in detail what component spectra can be resolved for various kinds of spectral overlap.

Materials and Methods

Asynchronous cultures of *Scenedesmus quadricauda* and *Chlorella pyrenoidosa* were grown at 23°C with continuous shaking in a modified Kessler's medium as described by Bishop and Senger [29]. Continuous illumination was provided by fluorescent lighting (General Electric 'cool white') at an intensity of 1 W/m^2. For measurements, algae were pelleted and resuspended in fresh growth medium.

Chloroplasts were isolated from market spinach by the method of Gross [30]. This procedure removes the outer chloroplast membrane, most mono- and divalent cations, and any cation binding sites unattached to membranes [31]. For all experiments, chloroplasts were resuspended in 100 mM sucrose, and the pH adjusted to 8.0 with a minimal amount of Tris. Final Tris concentration was about 0.1 mM.

A Spex Fluorolog I spectrofluorimeter, equipped with dual monochromators for both excitation and emission, was used for fluorescence measurements. Samples were thermostatted at 22°C in a 1 cm square cuvette, with the amount of photosynthetic material adjusted to give an optical density of 0.3 at 680 nm. This density was necessary to achieve a measurable fluorescence level at the longest excitation wavelengths.

To insure maximum variation in the relative contribution of PS I and PS II, excitation wavelengths covered the range from 650 to 710 or 720 nm. Over this range, exciting-light intensity varied from 0.05 to 0.10 W/m^2.

Some preliminary experiments were performed to help characterize the physiological state of the algae and chloroplasts. Chloroplasts, using any excitation wavelength, did not exhibit fluorescence induction, shown by a lack of change in emission yield at 685 nm. Gross and Hess [31] have shown that chloroplasts fluorescence is quite high in ultralow salt, indicating State I conditions [30]. Inactivation of the water-splitting apparatus of PS II in chloroplasts by treatment with Tris, as described by Yamashita and Butler [32], did not affect the 685 nm fluorescence yield. Therefore, we conclude that these chloroplasts, under weak illumination, were in State I at the F_0 level.

The algae, both *Scenedesmus* and *Chlorella,* exhibited a slight fluorescence induction under illumination by the spectrofluorimeter in the 650–700 nm region. When algae were heated to 50 °C for 10 min to inactivate the water-splitting apparatus of PS II, the fluorescence yield declined slightly. Therefore, the algae were near, but not at, F_0. Several recent reports argue that most in vivo fluorescence induction is due to changes in the redox level of Q, the electron acceptor of PS II, and not to State I–II transitions [33,34]. Since Satoh and Fork [34] have shown that dark-adapted *Scenedesmus* are in State II, and our light intensities were well below those used by others to induce state changes [33,34], the algae were probably in State II.

For factor analysis, data collection involved recording emission spectra at several different excitation wavelengths. Samples were preilluminated for 5 min with the appropriate excitation wavelength before an emission spectrum was recorded.

All slits in both monochromators were set for a bandpass of 5 nm. Excitation spectra were deconvoluted to correct for distortion introduced by this bandpass, and corrected for variation in exciting light intensity. Emission spectra were corrected for self-absorption and for the wavelength-dependence of the detection system.

A photon-counting gallium arsenide photomultiplier tube (RCA C31034-02) was used to measure light emission. Pulses from the discriminator were fed to the multi-channel scaling input of a Tracor NS-570A signal averager. Repeated scans were averaged to improve the signal-to-noise ratio under low fluorescence conditions. Spectra were transferred over a serial line to a small laboratory computer for initial processing, and then over a telephone line to a large computer for factor analysis.

Data from each emission spectrum was divided into five subgroups. The first subgroup was created by taking every fifth data point, beginning with the first data point. The second subgroup was created by taking every fifth data point, beginning with the second data point. The third, fourth and fifth subgroups were similarly constructed. Each of the five subgroups then contained one-fifth of the data, with no duplication of points. These separate subgroups were then analogous to five separate experiments, albeit at lower resolution than the original. After smoothing, correction and removal of any signal due to light scattering, factor analysis as outlined in the theory section was performed on each of the five subgroups. This procedure was adopted to give an estimate of the uncertainty in the original spectra, the singular vectors, and the solution spectra.

Examples of data before and after smoothing and correction are shown in Fig. 1. Fig. 1A shows the emission spectrum of spinach chloroplasts excited at 650 nm. The sharp spikes at approx. 645 and 655 nm are due to the beginning and end of the scatter peak caused by overlap of the excitation and emission monochromators. Values around 650 nm are close to zero because a shutter was closed to protect the photomultiplier tube. Fig. 1B shows the 95% confidence limits obtained by applying the *t*-test to independently smoothed and corrected curves from the five data subgroups. Fig. 1C and 1D show analogous spectra from excitation at 710 nm.

Singular value decomposition yields pairs of singular vectors, with one member of each pair being a function of excitation wavelength, and the other being a function of emission wavelength. As an example of this, Fig. 2 shows the three emission singular vectors of spinach chloroplasts which had the largest singular values.

Since the singular value decomposition was performed independently on each of the five independent data matrices, five separate sets of vectors were produced. The cross-hatched area on each of the vectors in Fig. 2 shows the 95% confidence limits obtained by applying the *t*-test to the corre-

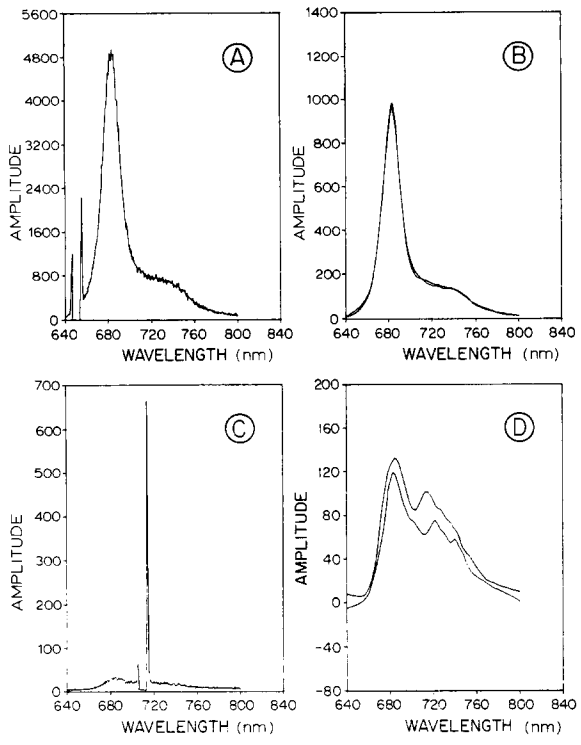

Fig. 1. Sample emission spectra of broken spinach chloroplasts before and after smoothing, removal of peak due to light scattering, and correction. (A) raw emission spectrum for 650 nm excitation; (B) smoothed and corrected version of (A) with 95% confidence limits of the emission band; (C) same as (A) except for 710 nm excitation; (D) same as (B) except for 710 nm excitation. The amplitude values are relative.

sponding sets of five vectors.

The first vector is well defined. The second is also fairly clean, though noisier than the first. However, the third vector has an uncertainty at all wavelengths which equals its average amplitude, so that the confidence limits bracket the X-axis. The third vector is mostly noise, and not usable in further analysis. These results mean that we were able to resolve only two components in this sample.

Once the number of resolvable components was determined, linear combinations of the significant singular vectors were taken to generate excitation and emission spectra. For two components, a particular choice of linear combinations is specified by identifying numerical values for the four elements of a 2 by 2 transformation matrix. Normalization of the height of the spectra leaves two elements to be defined. The values of these two elements were determined by a non-linear minimization routine which systematically varied the elements so that the resulting spectra differed minimally from known properties of the actual spectra.

Two principal criteria were used in this minimization: (1) all spectra must be nonnegative, and (2) the excitation and emission spectra for a particular component must obey the Stepanov relationship.

The Stepanov relationship states that the emission spectrum of an equilibrated excited state is

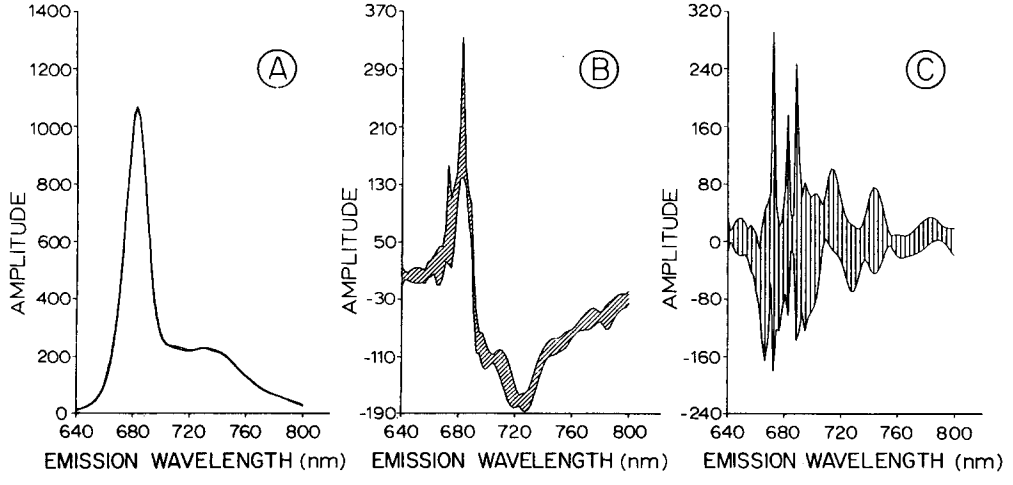

Fig. 2. First three emission singular vectors from factor analysis of spinach-chloroplast excitation-emission data. (A) first, (B) second and (C) third vector. Results indicate that only two independent components are discernable.

given by the absorption spectrum multiplied by the spectrum of a blackbody having the temperature of the vibrations, T [35,36]. For most molecules in solution, T is equal to the temperature of the solution. However, for some molecules, T is different (usually higher) than the ambient temperature. One rationale for this behavior is that the absorption and emission spectra are inhomogeneously broadened [37], possibly because the fluorescence lifetime is short compared to the orientational relaxation time of the solvent. In any event, for all cases of which we are aware, the Stepanov relationship has been found to hold for some T. It has previously been shown to apply with some accuracy to photosynthetic pigment complexes, with a T of room temperature [38,39].

Use of the Stepanov relationship can reduce the uncertainty in component spectra somewhat, and as a side benefit, indicates if equilibration has occurred during the molecules' excited state lifetime.

In some cases additional criteria were provided by fitting the excitation spectra to published action spectra for PS I and PS II.

The non-linear determination of the transformation matrix was performed independently on each of the separate sets of vectors produced from decomposition of the five independent data matrices, so that uncertainty in the determination of this matrix, as well as uncertainty in the determination of the singular vectors, is reflected in confidence bands attached to the final spectra (Figs. 5–7).

For further details, see Ref. 28.

Results

For both spinach and algae, the shape of the observed emission spectrum varied with excitation wavelength. An example of this is given in Fig. 1. Even with the low signal-to-noise ratio, it is apparent that with 710 nm excitation (Fig. 1D), fluorescence in the 710–740 nm region constitutes a larger fraction of the total emission than in the spectrum excited with 650 nm light (Fig. 1B).

As mentioned in the previous section, Fig. 2 indicates that only two components can be resolved for spinach chloroplasts.

Figs. 3 and 4 show the first three emission vectors obtained from the factor analysis of *Chlorella* and *Scenedesmus*. The first two vectors show that there are at least two components for each alga. The noise in the third vector of *Scenedesmus* is equal in magnitude to any signal that may be present; at best, resolution of three components is marginal. The third vector of *Chlorella* is more distinct, indicating three components with fair certainty.

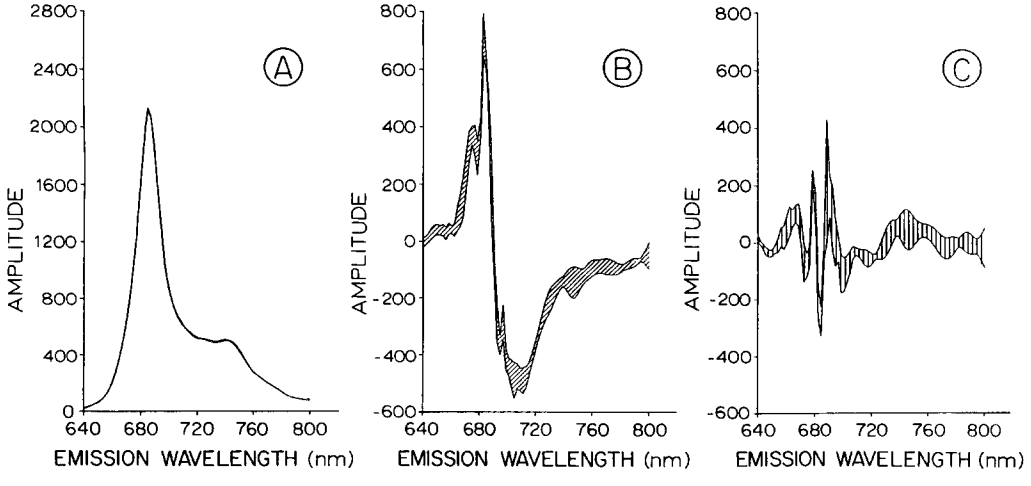

Fig. 3. First three emission singular vectors from factor analysis of *Chlorella* excitation-emission data. (A) first, (B) second and (C) third vector. Results show two components clearly, and suggest that a third is present.

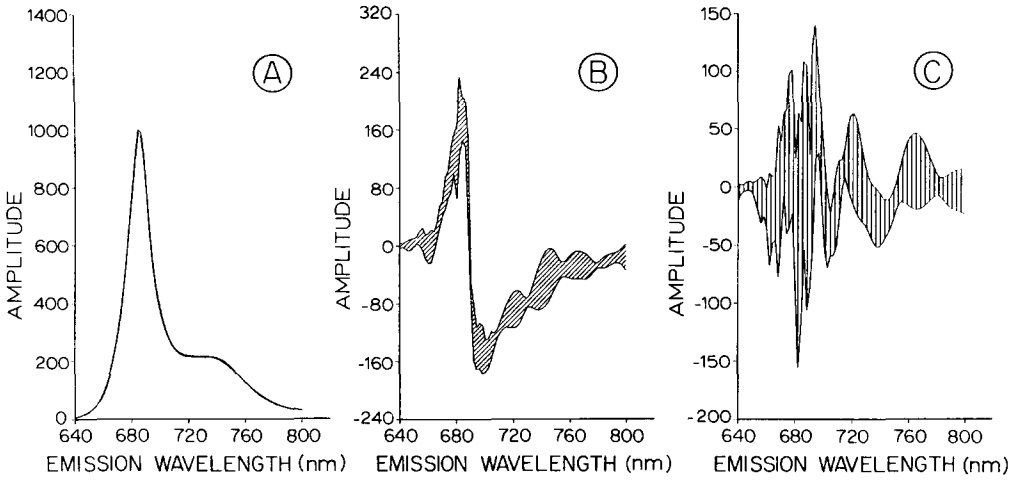

Fig. 4. First three emission singular vectors from factor analysis of *Scenedesmus* excitation-emission data. (A) first, (B) second and (C) third vector. Results show two components clearly, and a third marginally.

For spinach chloroplasts and the two algae, linear combinations were taken of the leading two singular vectors to obtain excitation and emission spectra for two components. Although the vectors from the algae were suggestive of a third component, the noise was too great to permit any useful determination of spectra for three components.

The resolved excitation and emission spectra of the two spinach chloroplast components are presented in Fig. 5. Both excitation spectra have maxima at 680 nm (resolution of 10 nm). One, which we call component 1, has a stronger absorbance at wavelengths longer than 680 nm. The other, component 2, has a stronger absorbance at 650 nm. Both components have emission maxima around 683 nm, but component 1 has a very pronounced long-wavelength shoulder.

The resolved component spectra of a two factor analysis of *Chlorella* and *Scenedesmus* are given in Figs. 6 and 7. The spectra for the two algae are very similar, but different from those of spinach chloroplasts. Again, we refer to the component with longer-wavelength absorption and emission as component 1, and the other as component 2. Component 2 shows an excitation maximum at 670 nm (again, resolution of 10 nm), with a shoulder at 650 nm.

There are readily apparent differences between

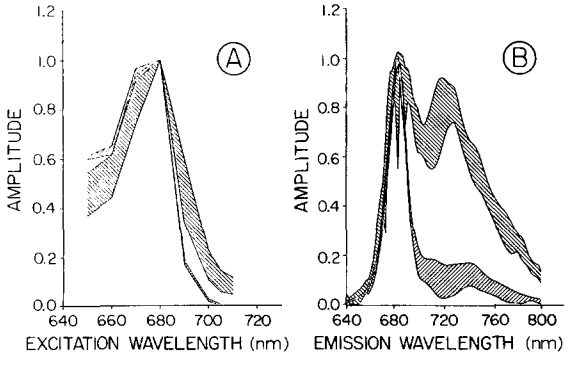

Fig. 5. Resolved excitation and emission spectra of spinach chloroplasts. The spectra with the same cross-hatch pattern originate from the same component.

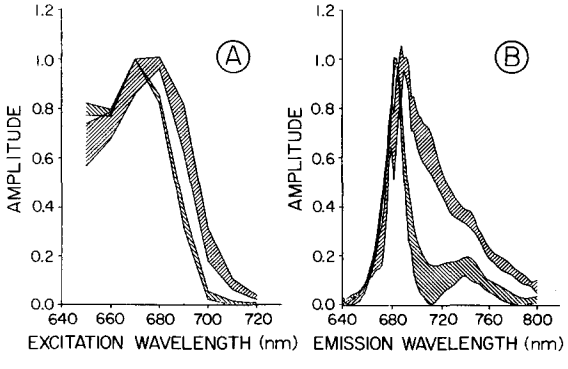

Fig. 6. Resolved excitation and emission spectra of *Chlorella*.

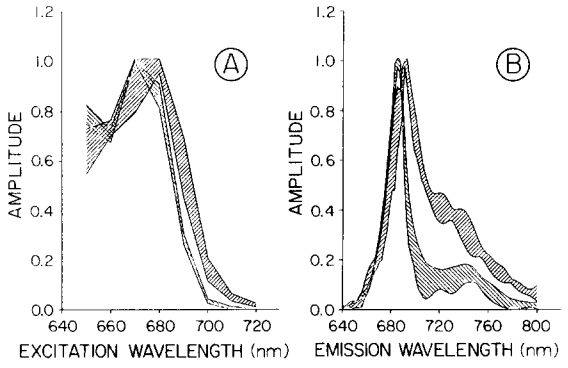

Fig. 7. Resolved excitation and emission spectra of *Scenedesmus*.

the resolved component spectra of algae and those of spinach chloroplasts. The most notable difference is in the amplitude of the 730 nm shoulder in the emission spectrum of component 1. Furthermore, component 1 emission peaks at 690 nm in algae, as opposed to 683 nm in spinach. Another significant difference is in the height of the 650 nm shoulder in the excitation spectra: component 2 has an absorbance at 650 nm which is approx. 60% of the peak in spinach, and approx. 80% of the peak in the two algae.

The determination of the transformation matrix, and the resulting spectra, was repeated with the additional constraint introduced by a least-squares fit between the component excitation spectra and published action spectra for PS I and PS II [38,40–44]. In all cases, the excitation spectrum of component 1 matched the action spectrum of PS I, and the excitation spectrum of component 2 matched the action spectrum of PS II, to within the estimated accuracy of the action spectra.

In the course of calculating the component spectra, the factor analysis program also computed the relative total emission for each component (for excitation at 680 nm), and Stepanov temperatures for each component. By scaling the two excitation spectra to obtain the best fit to the absorption spectrum, the program also calculated the relative absorbance and fluorescence quantum yield of each component. The ranges of values obtained from the five independent data subsets are listed in Table I.

Discussion

Factor analysis can provide a useful resolution of the room temperature fluorescence spectra of the photosynthetic apparatus into two components.

Our data gave no evidence for a third component in broken spinach chloroplasts, poor evidence

TABLE I

CHARACTERISTICS OF THE SPECTRAL COMPONENTS

All component determinations used nonnegativity of spectra and fluorescence quantum yields, and the Stepanov relationship between excitation and emission spectra, as criteria for selecting the transformation matrix. Where indicated, action spectra for PS I and PS II were fit to the excitation spectra as additional criteria. Total fluorescence, absorbance and quantum yield ratios are for component 2 relative to component 1. Total fluorescence is for excitation at 680 nm. Relative absorbance is at 680 nm. The last two columns refer to the Stepanov temperatures for components 1 and 2. Ranges shown are extrema for five independent subsets of data, and thus correspond to 94% confidence limits.

Action spectra	Total fluorescence ratio (2/1)	Absorbance ratio (2/1)	Quantum yield ratio (2/1)	T_1	T_2
Spinach					
None	1.3–3.1	0 –0.81	3.8–∞	303–460	278–306
Refs. 39–41	2.7–4.3	0.63–0.75	4.2–5.7	304–441	286–306
Ref. 42	4.7–9.3	1.5 –2.4	3.2–3.9	365–516	278–299
Chlorella					
None	0.7–1.1	0 –0.17	6.5–∞	301–327	287–306
Ref. 43	2.8–3.5	0.9 –1.1	3.0–3.5	332–368	295–313
Ref. 44	1.3–2.1	0.3 –0.5	4.0–5.8	322–347	294–316

for a third component in *Scenedesmus,* and modest evidence for a third component in *Chlorella.* Using the related technique of matrix rank analysis, which identifies only the number of components, Govindjee and Yang [18] found two components in spinach chloroplasts, and Williams et al. [19] found two components in *Chlorella.*

Comparison of the component excitation spectra with action spectra for PS I and PS II suggests that component 1 is composed mostly if not entirely of PS I, while component 2 is composed mostly if not entirely of PS II. Component 1 has the stronger far-red absorbance which is characteristic of PS I. Component 2 has the more pronounced shoulder at 650 nm, corresponding to the greater contribution of chlorophyll *b* in PS II.

The emission spectrum of spinach component 2 corresponds well with the spectra of PS II particles isolated by several groups [45–47].

However, the emission spectrum of spinach component 1 does not agree with the spectrum of PS I preparations. PS I preparations having a strong 730 nm emission at room temperature have a peak at 690 nm [6,16], while component 1 peaks at 683 nm. PS I preparations which have maxima at 683 nm lack the strong 730 nm shoulder [45,48,49].

Two explanations for this discrepancy have occurred to us. First, the 683 nm emission may be due to PS II which is a part of component 1. Perhaps component 1 corresponds physically to the pigments in the unappressed region, which is mostly PS I, but also contains some PS II [50]. Second, the emission spectrum obtained for component 1 may be correct for PS I as it occurs in vivo, and isolation of PS I causes the loss of the 683 and/or the 730 nm emitting pigment. Arnzten and co-workers [6,7] have demonstrated that a subunit of PS I is responsible for the 710–740 nm emission, and that this subunit can be separated from the reaction center, leaving the emission maximum at 690 nm. Light emitted at 683 nm may be due to LHC associated with PS I which is separated on isolation. Most isolated PS I particles with 683 nm emission maxima have high chlorophyll *a/b* ratios, indicating that little LHC is present. The 683 nm emission in isolated particles may be due to functionally-detached chlorophylls in the preparations [45,49].

Since factor analysis could reliably indicate the presence of only two components in any organism studied, LHC did not have any easily distinguishable behavior of its own. Presumably, this is because of efficient energy transfer from LHC to an attached photosystem, so that the LHC is not an independent component. Alternatively, the emission spectra of LHC and PS II may be very similar at room temperature, and beyond the resolution of our measurements.

The other principal method for determining independent fluorescent components is the enumeration of fluorescence lifetimes [51]. Picosecond pulse experiments have identified three lifetimes [52,53], with some indication of more than three components. Moya and co-workers [54,55], using phase-modulation techniques at liquid nitrogen temperatures, argue for up to seven lifetimes in spinach chloroplasts or whole algae.

More than one lifetime may originate from the same set of excited states. For example, two lifetimes may be associated with Photosystem II, one for prompt fluorescence of photons which never cause a charge separation, and the other from nanosecond-delayed fluorescence due to charge recombination of the primary donor and acceptor [52,55]. If a single pigment complex exhibits multiple lifetimes, then these kinetic components would have identical excitation and emission spectra, and be considered as one entity in the excitation-emission factor analysis performed in this report.

The data in Table I demonstrate that factor analysis has some ability to identify important parameters besides the spectra themselves. The fluorescence from component 1 is 20–40% of the total, in agreement with its identification as being composed largely of PS I. The relative absorbance of the two components is not well-defined, and so provides no assistance in identification of the two components.

The precise agreement of the Stepanov temperature of component 2 with the ambient temperature is strong evidence of good equilibration within and between the excited states of the pigment molecules which comprise this component. As component 2 is pure or nearly pure PS II, this is good evidence of equilibration of excitations in that photosystem. This is a significant finding, because excited-state equilibration is the key as-

sumption underlying the computation of the free energy generated by a photosystem [39,56].

On the other hand, the Stepanov temperature of component 1 is less well defined, and significantly above the ambient temperature. This suggests an absence of equilibration in this component. Component 1 may be a composite of pigment complexes which are individually equilibrated, but which we have been unable to resolve. Or, there may truly be a lack of equilibration in the excited state of PS I, which is a major portion of component 1. Such a lack of equilibration would not be surprising, given the short lifetime of the excited state of PS I [51]. If the excited state of PS I is not equilibrated, then new methods will have to be developed to compute the free energy of this photosystem.

In summary, we have used the statistical method known as factor analysis to determine the number of independently absorbing and emitting components observable in an intact photosynthetic system at room temperature, to resolve excitation and emission spectra of each component, and to estimate the relative absorbance, fluorescence quantum yield, and Stepanov temperature of each. We were able to resolve two components, with significant differences between spinach chloroplasts and intact green algae. This new analytical method allows us to observe spectral properties of chlorophyll-protein complexes as they occur in vivo without the use of liquid nitrogen temperatures, and with minimal assumptions about the shape of the component spectra.

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References

1 Thornber, J.P., Markwell, J.P. and Reiman, S. (1979) Photochem. Photobiol. 29, 1205–1216
2 Kaplan, S. and Arntzen, C.J. (1982) in Photosynthesis, Vol. 1. Energy Conversion in Plants and Bacteria (Govindjee, ed.), pp. 65–151, Academic Press, New York
3 Glazer, A.N. (1983) Annu. Rev. Biochem. 52, 125–157
4 Anderson, J.M. and Melis, A. (1983) Proc. Natl. Acad. Sci. USA 80, 745–749
5 Hodges, M. and Barber, J. (1983) FEBS Lett. 160, 177–181
6 Mullet, J.E., Burke, J.J. and Arntzen, C.J. (1980) Plant Physiol. 65, 814–822
7 Haworth, P., Watson, J.L. and Arntzen, C.J. (1983) Biochim. Biophys. Acta 724, 151–158
8 Thornber, J.P. and Barber, J. (1979) in Topics in Photosynthesis, Vol. 3, Photosynthesis in Relation to Model Systems (Barber, J., ed.), pp. 27–70, Elsevier, Amsterdam.
9 Haworth, P. and Melis, A. (1983) FEBS Lett. 160, 277–280
10 Horton, P. (1983) FEBS Lett. 152, 47–52
11 Telfer, A., Hodges, M. and Barber, J. (1983) Biochim. Biophys. Acta 724, 167–175
12 Butler, W.L. and Hopkins, D.W. (1970) Photochem. Photobiol. 12, 439–450
13 Butler, W.L. and Hopkins, D.W. (1970) Photochem. Photobiol. 12, 451–456
14 Leclerc, J.C., Hoaru, J. and Remy, R. (1979) Biochim. Biophys. Acta 457, 393–409
15 French, C.S., Brown, J.S. and Lawrence, M.C. (1972) Plant Physiol. 49, 421–429
16 Brown, J.S. and Schock, S. (1981) Biochim. Biophys. Acta 636, 201–209
17 Weber, G. (1961) Nature 190, 27–29
18 Govindjee and Yang, L. (1966) J. Gen. Physiol. 7, 595–614
19 Williams, W.P., Murty, N.R. and Rabinowitch, E. (1969) Photochem. Photobiol. 9, 455–469
20 Lawton, W.H. and Sylvestre, E.A. (1971) Technometrics 13, 617–633
21 Ohta, N. (1973) Anal. Chem. 45, 553–557
22 Warner, I.M., Christian, G.D., Davidson, E.R. and Callis, J.B. (1977) Anal. Chem. 49, 564–573
23 Malinowski, E.R. and Howery, D.G. (1980) Factor Analysis in Chemistry, Wiley Interscience, New York
24 Shrager, R.I. and Hendler, R.W. (1982) Anal. Chem. 54, 1147–1152
25 Golub, G.H. and Reinsch, C. (1970) Numer. Math. 14, 403–420
26 Chambers, J.M. (1977) Computational Methods for Data Analysis, Ch. 5, Wiley, New York
27 Lin, C.-H. and Liu, S.-H. (1978) J. Chin. Chem. Soc. 25, 167–177
28 Marchiarullo, M.A. (1983) Ph.D. dissertation, The Ohio State University, Columbus, OH
29 Bishop, N.I. and Senger, H. (1971) Methods Enzymol. 23, 53–66
30 Gross, E.L. (1980) Methods Enzymol. 69, 474–481
31 Gross, E.L. and Hess, S.C. (1974) Biochim. Biophys. Acta 339, 334–346

32 Yamashita, T. and Butler, W.L. (1968) Plant Physiol. 43, 1978–1986
33 Krause, G.H., Vernotte, C. and Briantais, J.-M. (1982) Biochim. Biophys. Acta 679, 116–124
34 Satoh, K. and Fork, D.C. (1983) Photochem. Photobiol. 37, 429–434
35 Stepanov, B.I. (1957) Dokl. Akad. Nauk SSSR 112, 839–841 (English trans.: Sov. Phys. Doklady 2, 81–84)
36 Ross, R.T. (1967) J. Chem. Phys. 46, 4590–4593
37 Van Metter, R.L. and Knox, R.S. (1976) Chem. Phys. 12, 333–340
38 Knox, R.S. and Van Metter, R.L. (1979) in Chlorophyll Organization and Energy Transfer in Photosynthesis, CIBA Foundation Symposia, Vol. 61 (G. Wolstenholme and D.W. Fitzsimons, eds.), Excerpta Medica, Amsterdam, p. 177–190
39 Ross, R.T. and Calvin, M. (1967) Biophys. J. 7, 595–614
40 Kelly, J. and Sauer, K. (1965) Biochem. 4, 2798–2802
41 Sauer, K. and Park, R.B. (1965) Biochem. 4, 2791–2797
42 Joliot, P., Joliot, A. and Kok, B. (1968) Biochim. Biophys. Acta 153, 635–652
43 Reid, A. (1972) Proceedings of the 2nd International Congress on Photosynthesis, (Forti, G., Avron, M. and Melandri, A., eds.), Vol. 1, pp. 763–772, Dr. W. Junk Publishers, Dordrecht, The Netherlands
44 Wang, R.T. and Myers, J. (1976) Photochem. Photobiol. 23, 411–414
45 Boardman, N.K., Thorne, S.W. and Anderson, J.M. (1966) Proc. Natl. Acad. Sci. USA 56, 586–593
46 Vernon, L.P., Shaw, E.R., Ogawa, T. and Raveed, D. (1971) Photochem. Photobiol. 14, 343–357
47 Satoh, K., Strasser, R. and Butler, W.L. (1976) Biochim. Biophys. Acta 440, 337–345
48 Satoh, K. and Butler, W.L. (1978) Plant Physiol. 61, 373–379
49 Telfer, A., Barber, J., Heathcote, P. and Evans, M.C.W. (1978) Biochim. Biophys. Acta 504, 153–164
50 Andersson, B. and Anderson, J.M. (1980) Biochim. Biophys. Acta 593, 427–440
51 Karukstis, K.K. and Sauer, K. (1983) J. Cell. Biochem. 23, 131–158
52 Haehnel, W., Nairn, J.A., Reisberg, P. and Sauer, K. (1982) Biochim. Biophys. Acta 680, 161–173
53 Gulotty, R.J., Fleming, G.R. and Alberte, R.S. (1982) Biochim. Biophys. Acta 682, 322–331
54 Moya, I., Mullet, J.E., Briantais, J.-M. and Garcia, R. (1981) Proceedings of the 5th International Photosynthesis Congress, (Akoyunoglou, G., ed.), Vol. 1, pp. 163–172, Balaban International Science Services, Philadelphia, PA
55 Moya, I. and Garcia, R. (1983) Biochim. Biophys. Acta 722, 480–491
56 Marchiarullo, M.A. and Ross, R.T. (1981) Biochim. Biophys. Acta 636, 254–257