Biochimica et Biophysica Acta, 1056 (1991) 317–320

ADONIS 000527289100072E

BBABIO 40245

BBA Report

Resolution of the fluorescence spectra of plant pigment-complexes using trilinear models

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(Received 22 August 1990)

Key words: Fluorescence; Factor analysis; Principal components analysis; Chlorophyll-protein complex; (Pea chloroplast)

The intensity of fluorescence from a pigment is separately linear in functions of excitation wavelength, emission wavelength, and any chemical treatment which alters overall fluorescence yield. This multiple linearity permits the use of an extension of principal components analysis to resolve overlapping spectra without the use of any additional information. The method is used to resolve the spectra of pigment complexes in pea thylakoids, using the concentration of Mg^{2+} as the chemical treatment variable. Two components could be resolved accurately. One has little or no dependence on Mg^{2+}; the other, with an excitation spectrum resembling LHC II, has a dependence on Mg^{2+} which follows the Hill equation with a binding constant of 0.4–0.6 mM and a Hill coefficient of 2.4–3.1.

Marchiarullo and Ross [1] used a technique known to spectroscopists as principal components analysis or factor analysis [2,3] to resolve approximate fluorescence excitation and emission spectra for Photosystems I and II of two green algae and a higher plant. While this technique achieved an unprecedented resolution of these photosystem spectra in situ, it suffers from an inherent weakness. In this kind of analysis, the final spectra deduced are linear combinations of orthogonal characteristic vectors. These orthogonal vectors can be calculated without the use of any extra information or assumptions about the properties of the spectra, but determination of the linear combinations requires additional information about the system. Some conditions used are known to be true with great certainty; one example is that these spectra must never be negative. However, the conditions known with confidence may not be sufficient to define a unique linear combination and thus to define unique spectra.

In this report we introduce the use of a three-variable extension of the method which yields unique spectra without requiring any additional information. The model used is known in the mathematical psychology literature as PARAFAC or CANDECOMP [4–6]. It is a specific form of three-mode factor analysis [7]. The method has been used in luminescence spectroscopy to resolve the spectra of components in the eluent from a chromatography column [8], to resolve spectra and decay kinetics of phosphorescence from metalloporphyrins [9–11], and to resolve fluoranthenes with phase-resolved fluorescence [12]. The method has been used on absorption spectra to resolve the chromophores in the protein plastocyanin [13]. We believe that this report is the first application to resolving the fluorescence spectra of components within a biological system.

Trilinear analysis depends upon the measured quantity being separately linear in functions of each of three different independent variables. Here we use the property that the fluorescence of a dilute specimen is linear in (1) extinction coefficient, a function of excitation wavelength, (2) relative fluorescence intensity, a function of emission wavelength, and (3) overall fluorescence yield, a function of chemical treatment.

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); LHC II, light-harvesting chlorophyll *a*/*b* protein complex associated with Photosystem II; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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The PARAFAC model used in this report makes no explicit provision for excitation transfer between pigment-complexes. In this model, two pigment-complexes between which there is partial excitation transfer are represented as three components: one component for each complex emitting energy absorbed by it and one component representing energy absorbed by one complex and emitted by the other.

Another mathematical method for the decomposition of fluorescence into components, used with time-resolved data, is known as *global analysis* [14]. As usually applied, it differs from our method in using only two independent variables (time and emission wavelength) and in requiring a specific functional form for the dependence of fluorescence intensity on one of these variables (a sum of a few exponentials for the time dependence). It was first used on photosynthetic pigments by Holzwarth et al. [15].

Our earlier work [1] used excitation and emission wavelength as the independent variables. Here we add the concentration of Mg^{2+} bathing thylakoid membranes as the third independent variable. We study thylakoid membranes because this is the simplest and most readily studied system in which the photosynthetic pigment-complexes are thought to retain all of the important properties of their environment in an intact plant.

Murata [16] observed a change in emission spectra measured at 77 K after Mg^{2+} was added to broken chloroplasts. Mohanty et al. [17] then observed the same phenomenon in room temperature emission spectra. Similarly, others [18–20] have observed magnesium-induced modifications of the excitation spectra of chloroplast fluorescence.

Mg^{2+} or an equivalent cation is required for stacking of the thylakoid membranes. Mg^{2+} has also been described as changing the fraction of light distributed to each photosystem, changing the probability of excitation transfer between photosystems, and changing the rate of non-radiative decay. The effects of Mg^{2+} appear to be due to its binding to chlorophyll-protein complexes, notably LHC II (reviewed by Ref. 21). Since Mg^{2+} has different effects on different pigment-complexes, Mg^{2+} concentration is a useful variable with which to begin an exploration of the ability of trilinear analysis to resolve chlorophyll fluorescence in situ.

Pea (*Pisum sativum*) leaves were harvested from 14-day-old plants grown on vermiculite with 14 h/day of illumination. Intact chloroplasts were isolated by rapid centrifugation from a low-salt medium [22] followed by centrifugation through a silica sol step gradient (40% Percoll) [23]. These chloroplasts were then stored on ice in the dark as a concentrated suspension in 0.33 M sorbitol, 10 mM KCl, 10 mM potassium phosphate, 10 mM Hepes (pH 7.6) for 3–7 h. Broken chloroplasts were prepared by adding an aliquot of the chloroplast stock to a much larger volume containing 10 mM KCl and 10 mM Tricine at pH 8.0. After 10 min, an aliquot of these broken chloroplasts was added to a much larger volume of medium, such that the final assay mixture contained a specific Mg^{2+} concentration between 0.02 and 5.0 mM, 0.10 M sorbitol, 10 mM KCl, 10 μM DCMU, 10 mM Tricine at pH 8.0, with absorbance at 680 nm equal to 0.02. This mixture was held on ice for 40–80 min, and then warmed to 20 °C and held for 20 min before starting data collection, which required 20 min. All measurements were completed within 9 h of harvesting the leaves and all measurements on a given sample were completed within 2.2 h of lysing intact chloroplasts.

Fluorescence was measured at each combination of 28 excitation wavelengths from 400 to 708 nm and 19 emission wavelengths from 652 to 792 nm, using an SLM SPF-500C spectrofluorometer (SLM Instruments, Urbana IL) under the control of a microcomputer. A Corning C.S. 0–51 glass filter was used in the excitation beam to block second-order spectra. Excitation and emission bandpasses were each 4 nm. The spectral sensitivity of the instrument was calibrated using a photodiode whose own spectral sensitivity was calibrated with a thermopile. Light-scattering artifacts were avoided by excluding all data for which the difference in excitation and emission wavelengths was less than 11 nm. The temperature of the specimen holder was between 20.4 and 20.7 °C. A more complete description of the equipment is in Ref. 24.

The data were transferred to a supercomputer and fit by non-linear least-squares to the model

$$\mu_{ijk} = \sum_{f=1}^{F} \epsilon_f(\lambda_i^{ex})\, \pi_f(\lambda_j^{em})\, \phi_f([Mg^{2+}]_k)$$

where the three multiplied expressions are the relative (1) absorbance of component f at excitation wavelength i, (2) fluorescence intensity of f at emission wavelength j, and (3) fluorescence yield of f at Mg^{2+} concentration k. Any variation in quantum yield, concentration, or average absorbance of a component at different concentrations of Mg^{2+} would appear in the third term. One version of our computational methods is described in Ref. 25.

This entire procedure was repeated with groups of plants grown and studied independently. The results from our two largest datasets, one with eight different concentrations of Mg^{2+} and one with nine, are described here. The resolution of these datasets into two components is shown in Fig. 1. The estimated fluorescence from a component at a specific excitation wavelength, emission wavelength, and Mg^{2+} concentration is the product of the corresponding points on the three graphs. The excitation and emission spectra are normal-

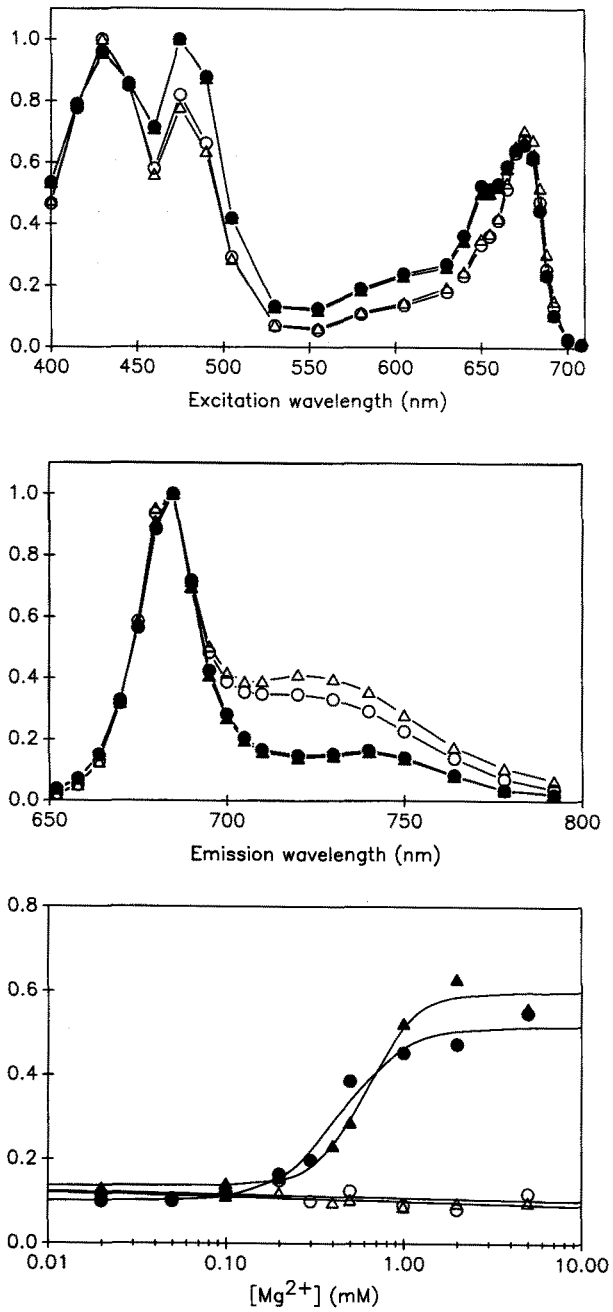

Fig. 1. Resolution of fluorescence into two components. Circles: dataset 1; triangles: dataset 2. Open symbols: Component 1; filled symbols: Component 2, resembling LHC II. Solid lines for Mg^{2+}-dependence of component 1 are the least-squares fits to a straight line; solid curves for Mg^{2+}-dependence of component 2 are the least-squares fits to the Hill equation.

ized to have a maximum of 1.0, so information about the relative magnitude of the two components is displayed in the $[Mg^{2+}]$ curves. The $[Mg^{2+}]$ curves for the two datasets have been scaled to have the same average value of log(amplitude).

Component 1 has some of the characteristics of Photosystem I and of pigment-complexes containing less chlorophyll *b*, including more emission at wavelengths longer than 700 nm and little or no dependence on Mg^{2+} concentration. Component 2 has some of the characteristics of Photosystem II and of pigment-complexes containing more chlorophyll *b*, including more absorbance at 475 and 650 nm due to chlorophyll *b*. Comparison of the excitation spectra with the absorption spectra of isolated chlorophyll-protein complexes [26] suggests that components 2 and 1 are more accurately identified as LHC II and 'other' than as Photosystems II and I. Component 1 may represent emission from the core complexes following either direct excitation or energy transfer from light-harvesting complexes. A weighted least-squares fit of the Mg^{2+} dependence of component 2 to the Hill equation gave $K = 0.44$ and 0.62 mM and $n = 2.4$ and 3.1 for datasets 1 and 2.

Systematic deviations between the data and the two-component model strongly suggest that there are more than two components, in agreement with the known complexity of pigment-complexes in thylakoids. However, resolution of the two datasets into three components did not give consistent results. Accurate resolution of three or more components will require even better data and new methods of data analysis, including the explicit treatment of excitation transfer between resolved components, which has not been considered in the analysis presented here.

We thank C.A. Breitenberger, E.L. Gross, and R.T. Sayre for advice on the preparation of our samples, and C.R. Ingling and X.Q. Wang for calibrating the spectral sensitivity of the fluorometer. This work was supported in part by grants to R.T.R. from the Gas Research Institute and the U.S. Department of Agriculture, by grants to S.E.L. and R.T.R. from the National Science Foundation (DMS-8805402 and DMS-8902265), and by computing resources made available by the Instruction and Research Computing Center of The Ohio State University and by the Ohio Supercomputer Center of the State of Ohio.

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