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EXCITATION SPECTRA OF CHLOROPHYLL FLUORESCENCE IN SPINACH AND BARLEY CHLOROPLASTS AT 4 K

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Excitation spectra of chlorophyll *a* fluorescence in chloroplasts from spinach and barley were measured at 4.2 K. The spectra showed about the same resolution as the corresponding absorption spectra. Excitation spectra for long-wave chlorophyll *a* emission (738 or 733 nm) indicate that the main absorption maximum of the photosystem (PS) I complex is at 680 nm, with minor bands at longer wavelengths. From the corresponding excitation spectra it was concluded that the emission bands at 686 and 695 nm both originate from the PS II complex. The main absorption bands of this complex were at 676 and 684 nm. The PS I and PS II excitation spectra both showed a contribution by the light-harvesting chlorophyll *a/b* protein(s), but direct energy transfer from PS II to PS I was not observed at 4 K. Omission of Mg^{2+} from the suspension favored energy transfer from the light-harvesting protein to PS I. Excitation spectra of a chlorophyll *b*-less mutant of barley showed an average efficiency of 50–60% for energy transfer from β-carotene to chlorophyll *a* in the PS I and in the PS II complexes.

Introduction

One of the advantages of low-temperature spectroscopy is the improved resolution due to band sharpening. This applies both to absorption and fluorescence spectra. For photosynthetic material an additional advantage is a considerable increase in the yield of chlorophyll and bacteriochlorophyll fluorescence that is observed upon cooling to liquid nitrogen temperature or below.

Low-temperature fluorescence spectroscopy of photosynthetic material has been mainly limited to the measurement of emission spectra (for reviews see Refs. 1 and 2). Although these spectra provide valuable information about the fluorescence properties of pigment-protein complexes, their interpretation in general has been hindered by a lack of adequate fluorescence action spectra. Action spectra of Chl *a* fluorescence in higher plant chloroplasts have been published amongst others by Murata et al. [3], Govindjee and Yang [4], Butler and co-workers [5,6], Menke and Schmid [7] and Gasanov et al. [8]; action spectra of intact algae by Cho and Govindjee [9], Sineshchekov et al. [10] and Goedheer [11]. The spectral resolution obtained in these measurements varied, but even the best spectra did not have enough resolution to take full advantage of the sharpening of the corresponding absorption bands that occurs upon cooling.

At low temperature, the fluorescence yield of chloroplasts is high. Thus, the signal-to-noise ratio of fluorescence action spectra should in principle be high enough to obtain spectral resolutions that are comparable to those of absorption spectra. In the present paper, we present action spectra measured at 4 K of Chl *a* fluorescence in spinach and barley chloroplasts and compare them with the corresponding absorption spectra of the same preparations.

Abbreviations: Chl, chlorophyll; PS, photosystem; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Materials and Methods

Chloroplasts from spinach and barley were prepared as described elsewhere [12], after grinding the leaves in a mortar or a blender. Spinach and barley were grown in the laboratory at about 22°C at about 3 000 and 8 000 1x, respectively. The chloroplasts were resuspended in 50 mM Tricine buffer, pH 7.8, containing 0.4 M sucrose, 10 mM KCl and 5 mM $MgCl_2$ (except for the experiment of Fig. 2) and stored in the dark on ice until use. Before the measurements the suspension was mixed with glycerol in a ratio of 4 : 5 (v/v) in order to prevent crystallization upon cooling. The final chlorophyll concentration used for the excitation spectra was 10 μg/ml.

Absorption spectra were measured as described elsewhere [13]. The measurements of the excitation spectra were performed on the same apparatus, except that it was equipped with a second monochromator. The monochromator used to obtain the excitation light was supplemented with a Schott UG 10, 2 mm and a BG 38, 2 mm filter for the blue region and with a BG 26, 3 mm filter for the red region of the spectrum. The sample was contained in a perspex vessel of 1 mm thickness. Fluorescence was detected at 90° to the excitation beam at the rear side of the cuvette, which was positioned at an angle of 45° to the incident beam. The fluorescence passed a second monochromator supplemented with appropriate cut-off (Schott RG) filters. The excitation monochromator was set at a half-bandwidth of 1.6 nm, and the analyzing monochromator was set at 3.2 nm. The apparatus was connected to an on-line computer system. The spectra were corrected for the intensity of the excitation light and were plotted as fluorescence intensity per incident quantum as a function of wavelength.

Results and Interpretation

Fig. 1 shows absorption and fluorescence excitation spectra of spinach chloroplasts, measured at 4 K. The emission spectrum (not shown) obtained upon excitation at 440 nm was similar to that reported by Rijgerberg et al. [14] with maxima at 735, 695 and 686 nm. The long-wave band in the emission spectrum is generally ascribed to PS I, the two other

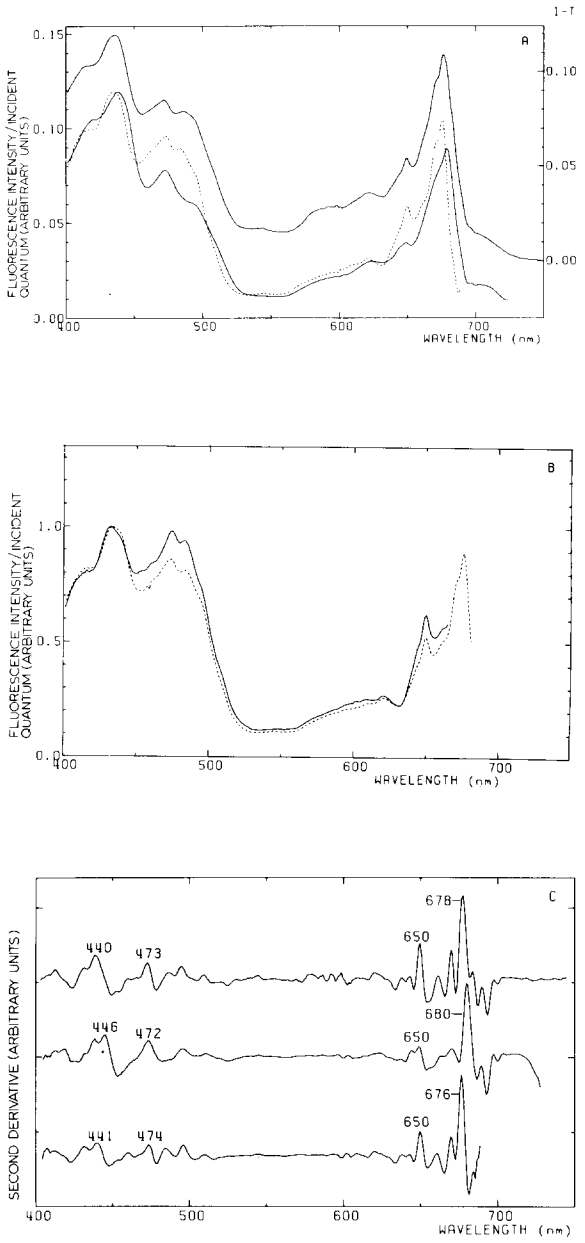

Fig. 1. Fluorescence excitation and absorption spectra of spinach chloroplasts, recorded at 4.2 K in the presence of Mg^{2+}. (A) Absorption spectrum and excitation spectra for fluorescence recorded at 696 nm (- - - - -) and 738 nm (———). Note that the absorption spectrum (———, upper) is shifted vertically with respect to the others. (B) Excitation spectra for the emission at 680 (———) and 687 nm (- - - - - -). (C) Second derivatives (inverted) of the absorption spectrum (top) and of the excitation spectra for 696 (center) and 738 nm (bottom) emission, plotted on an arbitrary scale.

TABLE I

BANDS IN THE ABSORPTION AND EXCITATION SPECTRA AT 4.2 K

The positions of the bands (nm) in the absorption (Abs) and excitation (Ex) spectra (for emission at the wavelength indicated) were obtained from the second-derivative spectra. Except for the bands at 467 and 666–668 nm of mutant barley, corresponding bands are displayed in the same column.

Spinach (+Mg^{2+})													
Ex 738:	472		496	509	650	663	671			680		690	697
Ex 696:	474	485	495	509	650	661	670	676			684		
Ex 687:	474	485	496	509	650	661	670	676					
Ex 680:	474	485	496	509	650	661							
Abs:	473	487	495	509	650	662	671		678		685	690	698
Spinach (–Mg^{2+})													
Ex 738:	474	485	496	510	650	661	670		678			690	
Ex 696:	475	485	496	510	650	661	670	676			684		
Barley wild type													
Ex 738:	475		497	510	649	661	671			681		691	698
Ex 696:	475	485	497	510	649	660	670	676			684		
Abs:	472	486	496	509	649	661	671		678		685	692	699
Barley mutant No. 2													
Ex 733:	467		496				666			682		691	697
Ex 696:	467		495			660	667	677			684		
Abs:	467		495			661	668		680		686	693	699

bands have been attributed to PS II [1,2,13]. In addition, there was a shoulder near 680 nm, presumably due to emission from Chl *a* belonging to the light-harvesting Chl *a*/*b* protein(s) [13,14].

The excitation spectra given in Fig. 1A and B are those of the emission at 680, 687, 696 and 738 nm. The overall shapes are similar to those of spectra obtained earlier at 77 K [3–8], but our spectra are more detailed and of higher resolution, and they are of about the same quality as the absorption spectrum (see Also Fig. 1C).

In order to determine the location of the bands in the absorption and excitation spectra, the second derivatives were determined (Fig. 1C). The bands, with the exception of those in the Soret region of Chl *a*, are listed in Table I. With a few exceptions that will be discussed later, there is an excellent correspondence between the absorption and excitation spectra. Many bands are common to all spectra determined. Nevertheless, as was to be expected, there are differences. The 738 nm excitation spectrum shows a clear preponderance of long-wave-absorbing Chl *a*, whereas the other spectra show a larger contribution by Chl *b* (474 and 650 nm) and by short-wave bands of Chl *a*. The Chl *a* bands at 662 and 670 nm appear to be common to all excitation spectra and are probably due to the light-harvesting Chl *a*/b protein(s), as will be discussed below.

For the 738 nm spectrum, the major band in the Q_y region is at 680 nm, and there are additional, weaker bands at 690, 697 and a broad band near 710 nm, which is not well resolved in the second derivative. The main peak in the Soret region of Chl *a* could be resolved in two bands, located at 435 and 445 nm (Fig. 1C).

The excitation spectra for 696 and 687 nm fluorescence were very similar. The highest peak was at 676 nm; the first spectrum showed in addition a weak band at 684 nm. In the Soret region main bands were located at 432 and 440 nm. The similarity between these two spectra strongly supports the notion that the 686 and 695 nm emission bands both originate from the PS II complex [13,14]. The excitation spectrum for the emission at 680 nm (Fig. 1B) shows the same bands as the PS II spectra, but the relative contribution of Chl *b* is clearly larger. All three spectra show a band at 485 nm that is absent in the PS I spectrum. The bands at 496 and 510 nm are probably due

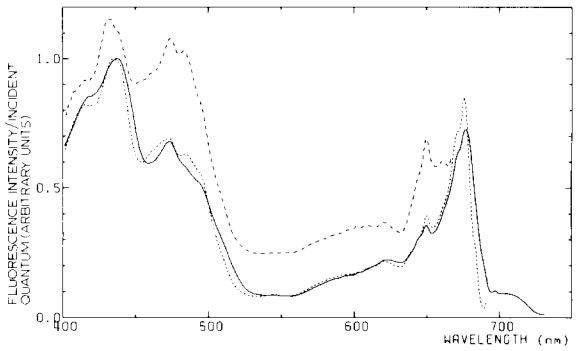

Fig. 2. Excitation spectra for the emission at 680 (– – –), 696 (- - - - - -) and 738 nm (———) in the absence of Mg^{2+}. The 680 nm spectrum is shifted vertically by 0.15 units to enhance clarity.

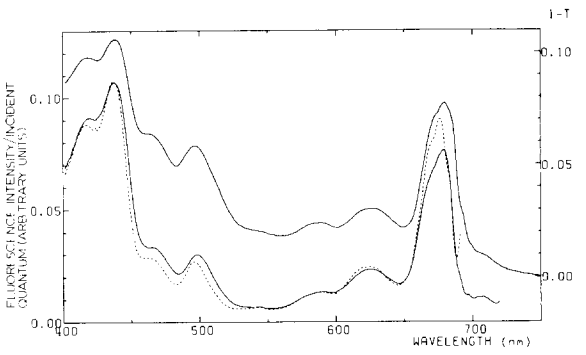

Fig. 3. Absorption spectrum (shifted) (upper trace) and excitation spectra for the emission at 696 nm (- - - - - -) and 733 nm (———) of chloroplasts from mutant No. 2 of barley.

to carotenoid. The PS I and PS II bands at 680 and at 676 nm, respectively, are apparently too close together to be resolved in the absorption spectrum and merge into one peak at 678 nm. A similar phenomenon is observed in the Soret region of Chl *a*.

Excitation spectra obtained in the absence of Mg^{2+} or other divalent cations are given in Fig. 2. Table I summarizes the positions of the maxima. The 738 nm spectrum now more resembles that obtained for the 696 or 687 nm emission. The main maximum in the Q_y region had shifted to 678 nm. The Chl *b* bands were relatively higher than in the presence of Mg^{2+}. The excitation spectrum for 696 nm emission was not basically altered by the absence of Mg^{2+}. The relative contribution of Chl *b*, of the Chl *a* bands at 670 and 661 nm and of the bands at 485 and 496 nm was decreased, however. In the region above 676 nm the PS II spectra with and without Mg^{2+} were the same.

Absorption and excitation spectra of barley mutant Chlorina No. 2 are shown in Fig. 3, and additional data on wild and mutant barley are presented in Table I. The absence of light-harvesting Chl *a*/*b* protein(s) is clearly reflected by the spectra of the mutant, while the spectra of the wild strain (not shown) were similar to those of spinach (see Table I). In the Q_y region of Chl *a*, the PS II spectrum of the mutant showed bands at 677, 667, 684 and 660 nm in order of decreasing amplitude. The 684 nm band was significantly higher, both in the absorption and in the PS II action spectrum, than in the corresponding spectra of the wild type. The PS I spectrum showed bands at 682, 665, 691, 697 and 707 nm. The band at 670 nm observed in the corresponding spectra of the wild type and of spinach chloroplasts was absent. Both spectra showed bands at 496 and 467 nm, presumably due to β-carotene [16].

Discussion

It is of some interest to compare our excitation spectra to those of others, measured at 77 K [3–8,17]. The older spectra [3,4] are not very detailed, and give no evidence for different contributions of short- and long-wave-absorbing chlorophylls to the excitation spectra of PS I and PS II. More recent spectra [5–8] show somewhat more detail. The excitation spectra of Kitajima and Butler [5] for the variable and 'base' fluorescence at 750 nm show maxima at 677 and 681 nm, respectively, and correspond fairly well with our excitation spectra for the 696 and 738 nm emission. Both spectra show additional bands at about 650 and 670 nm that were ascribed to the light-harvesting protein complex. Excitation spectra obtained by Gasanov et al. [8] for the 740 nm emission by spinach chloroplasts and a stroma PS I fraction showed maxima at 680 and 650 nm in the red region. The spectrum recently published by Menke and Schmid [7] for PS I fluorescence of a tobacco leaf peaks, however, at a relatively short wavelength

(673 nm). The PS I excitation spectra of chloroplasts from wild-type and mutant barley obtained by Boardman and Thorne [17], although less detailed, are similar to ours in the red region, but appear to be severely distorted below 600 nm. The prominent excitation band observed by these authors near 580 nm is totally lacking in our excitation spectra.

Excitation spectra of the green alga *Chlorella* [9] (see also Ref. 10) are in general agreement with our data for higher plant chloroplasts; they show a maximum at about 676 nm for PS II and one at somewhat longer wavelength for PS I emission.

The data presented here support some of the conclusions obtained earlier from emission spectroscopy and measurements of fluorescence kinetics at low temperature. The strong similarity between the excitation spectra for 686 and 695 nm emission are in agreement with the hypothesis that the emission bands at these wavelengths ('*F*-685' and '*F*-695') originate from the same pigment complex: the PS II complex [13,14]. It is conceivable that the small pool of Chl *a* species absorbing at 684 nm that can be observed in the excitation spectrum emits at 695 nm, whereas the larger pool absorbing at 676 nm may fluoresce at 686 nm (cf. Ref. 9). To explain these Stokes shifts of about 10–11 nm by means of the Kennard-Stepanov formalism [18–20], one has to use an effective temperature of about 25 K, rather than 4 K, as was also observed for the bacteriochlorophyll emission band in *Rhodospirillum rubrum* [13,21]. For this calculation we used as estimated bandwidth of 8–9 nm for the absorption bands, and assumed a Gaussian shape [22]. In a similar way the broad absorption band at 710 nm may be related to the broad PS I emission at 735 nm. As discussed elsewhere [13,23], the small pools of long-wave-absorbing Chl *a* would become strongly fluorescent at low temperature due to a shift in the temperature equilibrium between these pools and the bulk chlorophyll absorbing at shorther wavelength. For PS II the increase in the intensity at 686 nm below about 60 K [14] indicates that below this temperature the rate of energy transfer becomes too low to obtain such an equilibrium.

The high amplitude of the 684 nm band in the excitation spectrum of the 696 nm fluorescence of the barley mutant is in agreement with the larger emission at 696 nm of the mutant as compared to the wild type [13,14]. Apparently, this reflects the higher proportion of PS II complex (24% as compared to 10% in the wild type [15]).

The relatively high amplitude of the Chl *b* bands in the excitation spectrum for 680 nm emission supports the hypothesis [13,14] that '*F*-680' is due to the light-harvesting Chl *a*/*b* protein(s). The bands at 662 and 670 nm (and of course the Chl *b* band at 650 nm) in the excitation spectra for 696 and 738 nm may be ascribed to the light-harvesting complex. They are absent or very small in the absorption and excitation spectra of the barley mutant, and this is also true for the linear dichroism spectrum [24]. These bands correspond reasonably well with absorption bands of the isolated complex at room and liquid nitrogen temperatures [25–27]. The 677 nm band of the isolated complex may be hidden below more intense PS I and PS II bands near this wavelength. Some of the bands of the 696 nm excitation spectrum are absent in the excitation spectrum of the emission at 738 nm. This supports the conclusion [13,14] that at 4 K the extent of energy transfer from PS II and PS I is quite small, and that energy transfer from the light-harvesting Chl *a*/*b* complex to PS I occurs directly, not via PS II. The results obtained in the absence of Mg^{2+} are in line with earlier measurements done at higher temperatures [28–31] and indicate a stimulation of energy transfer from light-harvesting complex or PS II to PS I. The effects on the excitation spectrum for 696 nm emission indicate a decreased efficiency of energy transfer from light-harvesting complex to PS II, which may also explain the enhanced intensity of emission at 680 nm in the absence of Mg^{2+} [14].

In the carotenoid region, the excitation spectra of mutant barley show bands at 467 and 496 nm. These may belong to β-carotene [16] as was also suggested by the experiments of Goedheer [11] with extracted chloroplasts and Chl *b*-deficient algae. Comparison with the height of the corresponding absorption bands indicates an average efficiency of energy transfer from carotenoid to Chl *a* of about 50% within PS I and PS II. Gasanov et al. [8] obtained significantly lower efficiencies for fractionated PS I and PS II preparations from spinach. The excitation spectra of wild-type barley and spinach chloroplasts showed additional bands at 484 and 509 nm which are also present in the action spectrum of *F*-680 and

probably belong to carotenoid present in the light-harvesting Chl *a*/*b* complexes.

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