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LOW-TEMPERATURE SPECTRA OF CHLOROPLAST FRAGMENTS

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SUMMARY

Absorption and fluorescence–excitation spectra of chloroplast fragments, obtained by sonic oscillation and differential centrifugation, were measured at —196°. Several chlorophyll pigments, including C-705, were resolved in the low-temperature spectra. No fractionation of the chlorophyll pigments was achieved by differential centrifugation. The smallest chloroplast fragments (those in the supernatant of a 173000 × *g* centrifugation) contained the chlorophyll pigments in the same ratio as the intact chloroplasts. The fluorescence–excitation spectra showed that energy transfer between the pigments associated with the smallest fragments occurred to the same degree as in the intact chloroplasts. These results support the concept of a structural photosynthetic unit in which a C-705 molecule serves as an energy sink.

INTRODUCTION

The work reported here was undertaken to determine whether chloroplast fragments, obtained by sonic disruption and differential centrifugation, have the same pigment composition as the intact cell, particularly with regard to the small concentration of pigment absorbing near 705 mμ, C-705 (see ref. 1). Two considerations suggested these studies. First, if chloroplasts are made up of discrete photosynthetic units, as the work of THOMAS, BLAAUW AND DUYSENS[2], and PARK AND PON[3] indicates, the small units should contain all of the functional photosynthetic pigments. Second, it has been suggested, on mechanistic grounds, that the energy sinks, which C-705 molecules appear to be[1], might be located near the periphery of the grana[1,4,5] in contact with the water-soluble photosynthetic enzymes and substrates. If this were the case, the ratio of C-705 to chlorophyll *a* should vary with the size of the chloroplast fragment. The low-temperature spectroscopic measurements employed in the work reported here resolve several chlorophyll complexes, including C-705, which are not distinguishable from chlorophyll *a* after extraction.

METHODS AND MATERIALS

The techniques used in measuring absorption and fluorescence–excitation spectra of light-scattering materials have been described previously[1,6,7]. For spectral measurements at low temperature, the sample cell was placed in liquid nitrogen as shown in

Fig. 1. Fluorescence–excitation spectra were measured with a cut-off filter, which transmitted wavelengths longer than 730 mμ (Corning filters No. 2600, 9830 and 5030), between the sample and the phototube (EMI 9558). Absorption spectra were measured with the cut-off filter removed. The half-width of the monochromatic beam varied monotonously from 1 mμ at 500 mμ to 5 mμ at 750 mμ.

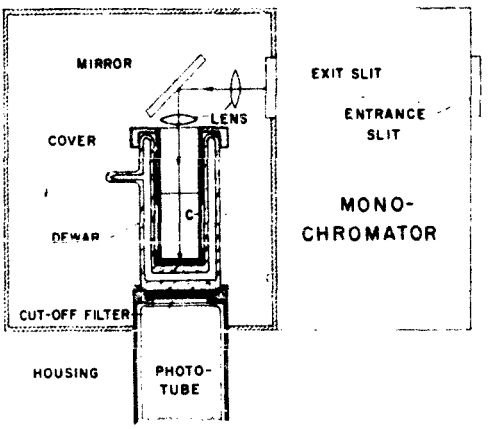

Fig. 1. Spectrophotometer with Dewar flask. The sample is placed in the metal-walled cell, C, which is surrounded by liquid nitrogen. The cut-off filter is used for fluorescence–excitation spectra.

Chloroplast fragments were prepared by disrupting spinach chloroplasts or algal cells in a Raytheon 10 kcycles sonic oscillator. The fragments were separated according to size by differential centrifugation in a Spinco preparative ultracentrifuge at speeds which gave maximum centrifugal forces of 20 000, 80 000, 145 000 and 173000 × *g*.

Chloroplasts were prepared from spinach by a method similar to that of PARK AND PON[8]. *Chlorella vulgaris*, var. viridis (chodat), was grown in shaker culture[8] to a concentration of 30–35 ml cells/l. *Euglena gracilis*, var. bocillaris, was grown in a similar manner but with a high organic medium[9].

RESULTS

Only the spectra for the supernatant fraction from the high-speed centrifugation and the total sonicate are shown. The spectra of fractions of intermediate-particle size (those sedimenting at 20000, 80000, 145000 and 173000 × *g*) were the same as the spectra which are presented. The absorption and fluorescence-excitation spectra of the sonicates were essentially identical with those of the intact cells with the exception that the absorption spectra of the intact cells were somewhat flattened due to the greater sieve effect[10].

Absorption and fluorescence–excitation spectra of chloroplasts from spinach are shown in Fig. 2. The total sonicate was diluted to approximately the same absorbancy at 680 mμ as the 173000 × *g* supernatant fraction. At the temperature of liquid nitrogen, the absorption spectra of these samples become much sharper and more intense. The sharpening is due to the depopulation of the higher vibrational levels at low temperature. The intensification results from the longer optical path due to the

increase of light scatter in the frozen sample[11]. At −196° the chlorophyll *b* band is clearly resolved and the main chlorophyll *a* band appears to be made up of two components. These two components have been resolved by FRENCH[12] in derivative spectra at room temperature.

The concentration of pigments in the supernatant from the 173000 × *g* centrifugation is not great enough to show the absorption band at 705 mμ directly. This band is shown in the fluorescence–excitation spectra at the top of Fig. 2. The fluorescence–excitation spectra for the emission of wavelengths longer than 730 mμ selectively enhance the absorption bands of components which fluoresce in the far-red region because most of the emission from these components is transmitted by the cut-off filter whereas only a small fraction of the chlorophyll *a* emission is transmitted to the phototube. The excitation bands cannot be used for quantitative estimates of the components. However, by keeping the concentration of pigments the same in the sonicate and the supernatant, the components in the two samples can be compared. The fluorescence–excitation spectra show that the concentration of C-705 is the same in both samples.

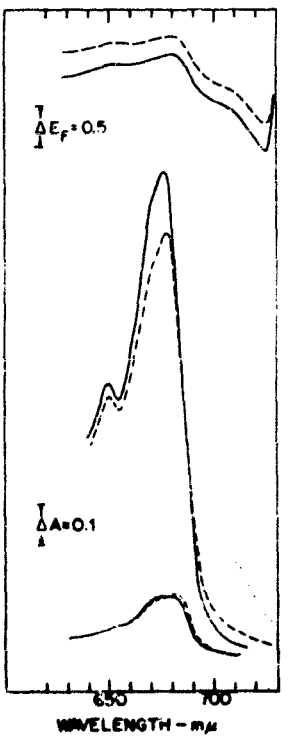

Fig. 2. Spectra of chloroplast fragments from spinach: – – – –, total sonicate; ———, supernatant from 173000 × *g* centrifugation. Lower curves: absorption spectra of 3-mm-thick sample at room temperature. Middle curves: absorption spectra of same samples at −196°. ....., absorption spectrum of supernatant concentrated 5 fold. Upper curves: fluorescence–excitation spectra at −196°.

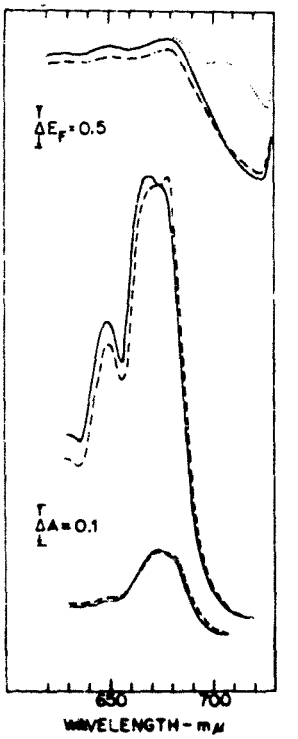

Fig. 3. Spectra of chloroplast fragments from Chlorella. – – – –, total sonicate; ———, supernatant from 173000 × *g* centrifugation. Lower curves: absorption spectra of 3-mm-thick sample at room temperature. Middle curves: absorption spectra of same samples at −196°. Upper curves: fluorescence–excitation spectra at −196°. ....., fluorescence–excitation spectrum of supernatant concentrated 10 fold.

The 705-mμ band can be observed directly if the suspensions are concentrated. The dotted curve in Fig. 2 is the absorption spectrum of the supernatant after a 5-fold concentration. An equal concentration of the sonicate gives an identical spectrum. At this concentration, however, the absorption spectrum is distorted in the high-density regions because the intensity of fluorescence excited by the incident light becomes comparable to the intensity of light transmitted by the sample.

The same spectral measurements on chloroplast fragments from Chlorella are shown in Fig. 3. Here at low temperature the main chlorophyll absorption band is clearly resolved into two components which may be designated C-670 and C-678, keeping in mind that the wavelength maxima are only approximate and may be shifted at low temperature. The 705-mμ component, however, is not seen in the fluorescence–excitation spectra of the dilute suspensions of the Chlorella chloroplast fragments. The 705-mμ-excitation band can be observed at —196° if the suspensions are concentrated 10-fold (dotted excitation spectrum in Fig. 3), but the 705-mμ band is not resolved in the low-temperature absorption spectrum of Chlorella even at these concentrations. Apparently less C-705 is present in Chlorella.

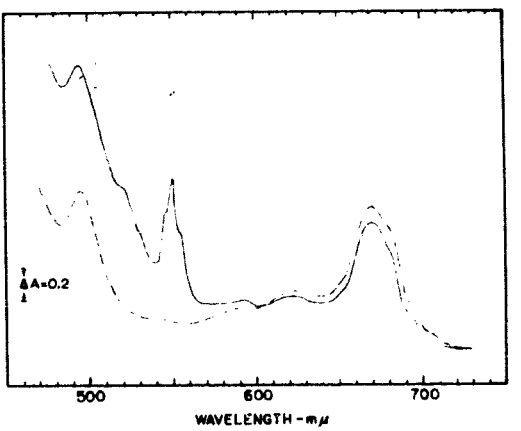

Fig 4. Absorption spectra of chloroplast fragments from Euglena at — 196°. ———, supernatant from 145000 × *g* centrifugation; – – – – –, total sonicate.

Both the room-temperature and low-temperature spectra of the Chlorella fragments show a small shift in the ratio of the components which make up the main chlorophyll band, the ratio of C-678 to C-670 being somewhat less in the supernatant fraction. This change occurs at a centrifugation of 30000 × *g*; the particles which sediment have an absorption spectrum identical with the spectrum of the total sonicate, whereas those in suspension have a spectrum identical with that of the 173000 × *g* supernatant. Little significance is attributed to this small compositional change. The chlorophyll which resides well within the larger chloroplast fragments may be somewhat richer in C-678. This same change in the ratio of the components which make up the main chlorophyll absorption band can be seen in the absorption spectra of chloroplast fragments from spinach as well (Fig. 2).

The spectra of the chloroplast fragments from Euglena in Fig. 4 show that the pigment composition of the fragments which remain in suspension at 145000 × *g*

is the same as the total sonicate. In contrast to these results, BROWN[13] has shown that, with chloroplast fragments from Euglena cells which have been aged for a week or more in a dark cold room, the derivative spectrum of the supernatant fraction from a 145000 × *g* centrifugation is somewhat different from that of the sedimenting fraction. Very little chlorophyll *b* is present in Euglena and an additional band described by FRENCH[11], is apparent at 695 mμ. The fact that the fluorescence–excitation spectra of spinach and Chlorella show the 705-mμ band, but not the 695-mμ band, indicates that the 695-mμ component is not a ubiquitous pigment.

The absorption spectrum of the supernatant fraction shows relatively large amounts of soluble cytochromes. The absorption band in the 550-mμ region is probably made up of two cytochromes; one with an absorption maximum at 550 mμ and shoulder at 546 mμ and another with an absorption maximum at 555 mμ. The carotenoid absorption band at 495 mμ is probably associated with the chloroplast fragments.

DISCUSSION

The spectra show that even the smallest particles obtained by sonic disruption of the chloroplasts contain all of the photosynthetic pigments in the same ratio as the intact cell. The fluorescence–excitation spectra are important because they depend, in part, on the transfer of excitation energy between the different pigments. In an intact leaf, the fluorescence–excitation spectrum at −196° for wavelengths longer than 730 mμ is due primarily to emission from C-705 (see ref. 1). Absorption of light by chlorophyll *b*, or either of the two forms of chlorophyll *a*, leads to emission from C-705, thus showing transfer of excitation energy to the longest wavelength component. The similarity between the fluorescence excitation spectrum of the supernatant fraction and that of the total sonicate or of the intact cells shows that energy transfer occurs between the different pigments associated with the very smallest fragments to the same extent as in the intact chloroplasts. Since energy transfer would not occur at these pigment concentrations unless the pigment were bound to the same particle, we can conclude that even the very small particles which remain in suspension at 173 000 × *g* contain all of the photosynthetic pigments in the proper ratio. (Absorption spectroscopy alone would not prove that the different pigments were on the same particle.) In the case of spinach, where the 705-mμ-absorption band can be measured, we can estimate the ratio of chlorophyll *a* to C-705 to be in the order of 100:1 on the assumption that the extinction coefficients are approximately equal. Chlorella has less of the C-705 and Euglena has an additional pigment absorbing at 695 mμ which may trap more of the excitation energy than C-705.

C-705 is undoubtedly the same pigment that KOK calls P_{700}. KOK[14] also tried to enrich chloroplast fractions in P_{700} by sonication and differential centrifugation and obtained the same results that we have. He found that the activity of P_{700} (as measured by a reversible bleaching by light or oxidizing agents) per unit chlorophyll was the same in the high-speed supernatant as it was in the sedimenting fraction.

The concept of a photosynthetic unit, first proposed by EMERSON AND ARNOLD[15], has found support from a number of investigations. THOMAS *et al.*[2] measured the Hill reaction with very small chloroplast fragments and concluded that the activity decreased sharply when the fragments were smaller than 10^6 Å^2. These minimum-sized particles were estimated to contain approximately 100 chlorophyll molecules.

PARK AND PON[3] showed that fragments of lamellae, approx. 800 Å across and 160 Å thick, which sedimented between 110000 and 145000 × *g*, had the full photochemical capacity for photosynthesis. They concluded that these fragments were aggregates of several fundamental units, spheroidal particles with major axes of 100 and 200 Å to which the name, "quantasome"[16], has been given. The results of the present paper provide further support for the concept of a photosynthetic unit by showing that chloroplasts can be broken up into very small particles which contain all of the various forms of chlorophyll in the same ratio as the intact cell. In addition, we must conclude that the C-705 trapping centers are located on the quantasomes and are uniformly distributed throughout the lamellae rather than being located near the periphery of the grana.

After the work reported here was completed, SAUER AND CALVIN[17] demonstrated electric dichroism with suspensions of quantasomes and showed that the dichroism was due to a small amount of a pigment absorbing near 700 mμ rather than to the main bulk of the chlorophyll *a*. The dichroic nature of the far-red absorption band had been previously demonstrated with intact chloroplasts[5,18]. Their work also shows that the long-wavelength absorbing chlorophyll molecules are on the individual quantasomes.

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