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DISTRIBUTION OF VARIABLE FLUORESCENCE AMONG
SUBCHLOROPLAST FRACTIONS

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SUMMARY

Spinach chloroplasts were fragmented by the French press technique and were separated by fractional centrifugation. Variable fluorescence was assayed by adapting a Cary 14 spectrophotometer for use as a modulated detector. The amount of variable fluorescence was directly related to the abundance of Photosystem II in the fractions. The 160K fraction which possesses negligible Photosystem II activity also possesses negligible variable fluorescence. The yield of background fluorescence from the 160K fraction is also much less than that from the other fractions. The presence of inactive chlorophyll in the 160K fraction cannot be explained by damage of Photosystem II during French press fragmentation.

INTRODUCTION

Stroma lamellae from chloroplasts appear to be entirely Photosystem I in character, both with respect to biochemical activity and chemical composition¹. In the previous paper we showed that while the quantum yield of NADP⁺ reduction by stroma lamellae is 1.0 at 710 nm, the efficiency at shorter wavelengths is much less². This ineffective absorption accounts for more than 50 % of the chlorophyll present in stroma lamellae.

In this paper we report the distribution of variable fluorescence³ among chloroplast grana and stroma lamellar fractions to determine whether the large amount of inactive chlorophyll in the 160K fraction is the result of a damaged Photosystem II.

METHODS

Chloroplast fractions were prepared from spinach using French press fragmentation followed by fractional centrifugation¹. The fractions studied were: original chloroplasts before passage through the French press, the whole homogenate obtained after passage through the French press and fractions precipitated at $1000 \times g$ 10 min (1K fraction), $10000 \times g$ 20 min (10K fraction), $40000 \times g$ 30 min (40K fraction), $70000 \times g$ 30 min (70K fraction). The 70K suspension was either precipitated at $160000 \times g$ 45 min (160K fraction) or was observed directly.

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

Relative fluorescence yields of the fractions at 680 nm were measured on a Perkin-Elmer MPF-2A fluorescence spectrophotometer with an R136-F photomultiplier tube and Corning 2-59 filter on the emission side of the sample. A 10-nm actinic beam at 436 nm, passing through a Corning 4-96 blue filter, excited fluorescence which was observed at 680 nm using a 10-nm band width. Samples were adjusted to 0.1 *A* at 436 nm, and were observed in a 1-cm cuvette. Fluorescence yield was proportional to chlorophyll content at this or lower concentrations. Addition of dithionite, as a reductant for the quencher Q, to the original chloroplast fraction gave no increase in fluorescence signal, indicating that the exciting beam of this single beam instrument was of sufficient intensity to reduce Q and saturate variable fluorescence. An 0.8 M Tris treatment of the original chloroplast fraction reduced the fluorescence yield by about 50 %, indicating that variable fluorescence was contributing about half the fluorescence intensity at 680 nm. This yield of variable fluorescence is slightly less than that reported by YAMASHITA AND BUTLER^{4,5}.

Variable fluorescence was assayed directly, using a Cary 14 spectrophotometer equipped with a % T slide wire, a high intensity light source, and an RCA 6911 photomultiplier shielded from the analyzing and actinic beams by a Corning 4-77 filter overlying a 2-58 filter. A means of actinic illumination was provided through a side port⁶. The Cary 14 was operated most successfully as a single beam instrument in the reference mode. The 30 cycles/sec modulated analyzing beam from the Cary 14 monochromator was set at 436 nm. The chloroplast sample, previously adjusted to 0.1 *A*, was then introduced in a 1-cm cuvette. At this absorbance fluorescence intensity was linear with concentration. The slit was initially adjusted by placing a sample in the reference side and operating in the double beam mode. This opening was then manually set for operation in the reference mode. The zero level of fluorescence was recorded in the absence of a sample. A continuous actinic beam at 436 nm was provided by a Bausch and Lomb 500 nm grating monochromator through a 5-58 blue filter. An increase in signal indicated an increase in the yield of fluorescence excited by the modulated beam. Several observations were made to check the validity of this procedure. To eliminate the possibility that the modulated beam was of sufficient intensity to excite variable fluorescence its intensity was measured and the actinic beam reduced to equal the intensity of the modulated beam. Under these conditions no detectable increase in variable fluorescence could be seen upon illuminating the sample with the actinic beam. To eliminate the possibility that the actinic beam was influencing the modulated detection system, the sample was replaced by a suspension of polystyrene latex spheres. Maximum illumination with the actinic 436-nm beam yielded no measurable increase in signal. Similarly, chloroplast samples reduced with dithionite showed no increase in fluorescence when illuminated with a high intensity actinic beam at 436 nm.

Also contributing to our confidence in this technique were the observations that variable fluorescence was destroyed by Tris treatment⁴ and by heating⁷. It was reduced by adding NADP⁺ and the cofactors for NADP⁺ reduction to the sample. The reduction in variable fluorescence brought about by addition of NADP⁺ and cofactors was reversed by $1 \cdot 10^{-5}$ M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU).

RESULTS AND DISCUSSION

Fig. 1 is a summary of data obtained for relative fluorescence yields of the fractions at 680 nm. The sample is first observed with no additions or further treatment.

The fluorescence yield under this condition consists of background plus light induced fluorescence and is represented by the clear areas.

Darkened areas represent a further increase in fluorescence yield obtained upon addition of dithionite to the sample. The relative amounts of maximum fluorescence in these fractions are closely related to the abundance of Photosystem II. The maximum fluorescence yield of the grana lamella (10K fraction) is 10 times greater than that of the stroma lamella (160K fraction) which correlates well with the distribution of Photosystem II in these two fractions. These data confirm the results of MICHEL AND MICHEL-WOLWERTZ^{8,9} and in addition show that the ability of chloroplasts to photoreduce Q is decreased by passage through the French press. Q itself does not appear to be destroyed by the treatment since the fluorescence yield of the French Press fraction upon addition of dithionite returns to the same level as that observed in the original chloroplasts.

In agreement with the observations of YAMASHITA AND BUTLER^{4,5}, Tris treatment greatly reduced the fluorescence yield of the original chloroplasts. The degree to which Tris reduced the fluorescence yield in the fractions was closely related to the abundance of Photosystem II in the fraction, the 10K fraction showing maximum reduction and the 160K fraction showing very little reduction.

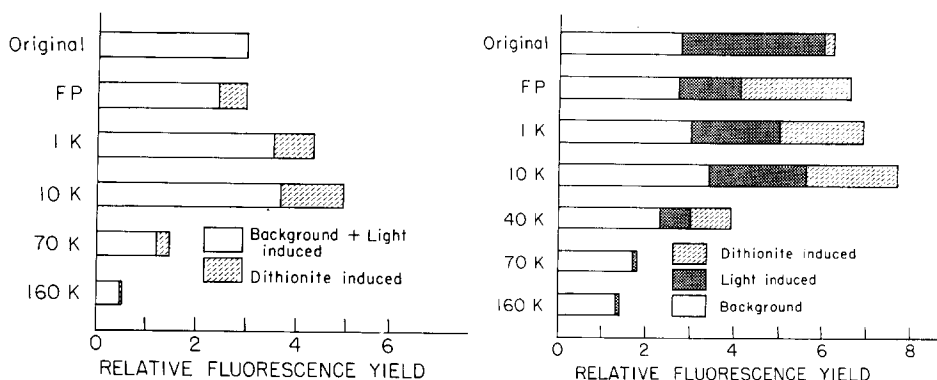


Fig. 1. Relative fluorescence yields of spinach chloroplast and chloroplast fractions measured at 680 nm. Actinic beam saturated the light-induced variable fluorescence. Increased fluorescence obtained upon addition of dithionite is represented by shaded areas. FP, French Press fraction.

Fig. 2. Relative fluorescence yields of spinach chloroplasts and chloroplast fractions measured using a modulated measuring beam. Background fluorescence is represented by clear areas, the light induced variable fluorescence by dotted areas and the additional fluorescence obtained upon addition of dithionite is represented by the slashed areas.

The contribution of variable fluorescence to total fluorescence was estimated using a modulated analyzing beam as described under METHODS. These results are given in Fig. 2. The fluorescence excited by the low intensity modulated beam in the absence of actinic illumination or added dithionite was considered the background fluorescence. The increase in fluorescence yield of the modulated beam upon actinic illumination was considered as light induced variable fluorescence. The increase in fluorescence yield of the modulated beam upon addition of dithionite was considered to be the total variable fluorescence. In the latter experiments fluorescence levels

were recorded 1 min after dithionite addition. The actinic beam had almost no effect on fluorescence yields when dithionite was present in the reaction mixture. The data in Fig. 2 show that background fluorescence is about 41–45 % of the total fluorescence in the original, French Press, 1K and 10K fractions. The proportion of background fluorescence rises to more than 90 % in the 70K and 160K fractions which are greatly depleted in Photosystem II. Again, Tris treatment almost totally destroyed the ability of the chloroplast fractions to photoreduce Q, whereas the level of background fluorescence was only slightly reduced.

While the background fluorescence can be measured directly using the Cary 14 as described here, the filter system used to shield the photomultiplier passes primarily wavelengths greater than 695 nm. Fluorescence emission spectra of the 10K and 160K fractions at room temperature show that the ratio of 710 nm/680 nm emission by the 160K fractions is twice as great as that by the 10K fraction. This is in agreement with observations by BOARDMAN *et al.*¹⁰ and by VREDENBERG AND SLOOTEN¹¹, who used digitonin separated subchloroplast fractions. This fact in combination with the emphasis on long wavelength spectral response by the filter system tends to minimize the fluorescence yield differences between the 10K and 160K fractions using the Cary 14 method, both with respect to total and background fluorescence. While the Cary 14 method serves to differentiate between background and variable fluorescence, an absolute comparison of total yields is better obtained from the fluorescence spectrophotometer as shown in Fig. 1.

The results given in Fig. 1 and 2 show that the 160K fraction, which is totally inactive in Photosystem II, demonstrates negligible variable fluorescence even in the presence of dithionite. Destruction of Q during French Press treatment cannot account for the absence of Q in the 160K fraction since the total abundance of Q does not appear to be reduced by French Press treatment. Therefore the inactive chlorophylls in the 160K fraction² do not appear to have resulted from damaged Photosystem II. Alternatively, they may be related to sites of membrane synthesis in the chloroplast².

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