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Inner structure of intact chloroplasts observed by a low temperature laser scanning microscope

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Abstract

Inner structure of isolated intact chloroplasts was observed for the first time by a method of laser scanning microscopy at the temperature of liquid nitrogen at 77 K. The microscope, based on gradient index optics, has a maximum resolution of 440 nm at the wavelength of 650 nm. Chloroplasts were excited into the Q-band of chlorophyll *b* by a krypton laser line at 647.6 nm and fluorescence was detected using two different interference filters. The 680 nm interference filter detects the regions where photosystem (PS) 2 mainly occurs, the 730 nm interference filter detects domains with predominant location of PS1. Since PS1 occurs mainly in stroma lamellae, whereas PS2 occurs mainly in grana regions we were able to view the structure of thylakoid membrane in isolated intact chloroplast that is the closest to *in vivo* state.

Additional key words: chlorophyll fluorescence; grana; photosystems 1 and 2; *Spinacia*; thylakoid.

Introduction

The organisation of thylakoid membranes in the chloroplast may be deduced from a 3-dimensional reconstruction of subsequent ultra-thin sections (Paolillo 1970). However, the procedure of plant tissue preparation for electron microscopy may affect the final result and the reconstructed thylakoid membrane organisation may differ from its *in vivo* state. The freeze-fracture methods can more realistically refer about the thylakoid membrane organisation but only in a very limited area of the chloroplast.

In this work a method of low temperature fluorescence microscopy and submicrometer-scale spectroscopy has been used to investigate the organisation and ultrastructure of chloroplast thylakoid membrane. Using the advantage of the laser scanning microscopy with the gradient index optics we distinguished the objects with a resolution less than 0.5 μm. Using this approach we observed, for the first time, the grana and stroma lamellae domains of the thylakoid membrane in intact chloroplast.

Materials and methods

Chloroplast preparation: Chloroplasts were prepared from fresh young leaves of spinach (*Spinacia sativa* L.). Leaves were suspended in isolation medium containing 50 mM Na_2HPO_4 (pH 7.5), 10 mM $MgCl_2$, and 0.4 M sucrose, and homogenised by five short (3 s) pulses in a blender. The homogenate was passed through 12 layers of a cotton mull and the filtrate was centrifuged for 5 min at 2 000×*g*. The pellet was gently re-suspended in the isolation buffer and the chloroplast suspension was immediately used for further analysis.

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Abbreviations: Chl – chlorophyll; IF – interference filter; LHC1 – light-harvesting complex of photosystem 1; LHC2 - light-harvesting complex of photosystem 2; PS – photosystem.
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Isolation of subchloroplast particles: The subchloroplast particles were isolated by centrifugation in 0-1.2 M sucrose density gradient prepared in a buffer containing 50 mM MES, 10 mM $CaCl_2$, and 0.02 % dodecylmaltoside at pH 6.5. Thylakoid membranes were solubilised in 1 % dodecylmaltoside and immediately loaded on the gradient. The gradient was run on a *Hitachi* β90 ultracentrifuge (*Hitachi*, Japan) for 13 h at 100 000×*g* in a fixed angle rotor *P70AT* (*Hitachi*, Japan). Bands containing PS1 and PS2 were collected by a syringe and stored at -65 °C.

Fluorescence spectra were recorded on a *SPEX Fluorolog* (*Spex*, USA) spectrofluorometer at the temperature of 77 K. Samples were immobilised on a home made holder and immersed directly into liquid nitrogen. Chloroplasts were excited at 470 nm and the fluorescence was detected in a range of 620–780 nm. The chlorophyll (Chl) concentration was adjusted to approximately 10 g m^{-3}.

Optical microscopy: Sample was diluted in isolation medium and observed in a reflection mode by conventional optical microscope (*Union Tokyo*, Japan, objective lens 40×). The image was detected and recorded by a CCD camera (*PentaMAX, Princeton Instruments*, USA).

The laser-scanning microscope is based on the rod-shaped radial gradient index microlens (*SELFOC®*, *Nippon Sheet Glass*, Japan) and it is described in Vacha *et al.* (1999). For the *SELFOC* lens, the refractive index changes perpendicular to the optical axis. A parallel laser beam falling on the input plane of the *SELFOC* lens is focused into a diffraction-limited spot on the output surface of the lens. Numerical aperture of the lens is increased to 0.9 by the solid immersion effect.

The laser beam was led and focused on the *SELFOC* lens by a set of lenses and mirrors. A scanning mirror driven by direct current motor actuator *Newport 850 F* (*Newport*, USA) together with two steering lenses changes the angle at which the laser beam enters the *SELFOC* lens and thus controls the position of the laser spot on the opposite side of the *SELFOC* lens where the sample is located. The fluorescence emission from the spot was detected by a cooled photomultiplier (*Hamamatsu R943-02*, Japan) and a *Stanford SR400* (*Stanford*, USA) photon counter. The photomultiplier tube was protected from the reflected laser radiation by *RG 665* or *RG 715* cut-off filters (*Schott*, Germany).

Laser line of 647.6 nm was provided by a krypton ion laser *Spectra Physics 165* (*Spectra Physics*, USA), amplitude stabilised, and focused on a microscope entrance pinhole. The *SELFOC* lens was mounted in a holder, chloroplast suspension was applied between the output surface of the lens and a microscope cover glass, and the holder was immersed in a cryostat filled with liquid nitrogen. Two-dimensional images were obtained by *x-y* raster scanning of the scanning mirror.

Results and discussion

Fig. 1 shows the image of isolated chloroplasts obtained by conventional optical microscope in a reflection mode. The suspension of chloroplasts was diluted by isolation medium to give a monolayer of chloroplasts that are well separated from each other. The majority of isolated chloroplasts had well-preserved lentil shape, approximately 5 μm in diameter.

Fig. 2 shows the 77 K fluorescence emission spectra of chloroplast suspension (*solid line*), a complex of PS2 with LHC2 (*dashed line*), and complex of PS1 with LHC1 (*doted line*) excited at 470 nm in Chl *b* of the light-harvesting complex of PS2. At the temperature of liquid nitrogen, the fluorescence emission of PS2 is in the wavelength region of 675–700 nm with a broad weak vibration band at 730 nm. The fluorescence of PS2 is composed of the emission of LHC2 peaking at 681 nm (Hemelrijk *et al.* 1992) and the emissions at 685 and 695 nm which belong to the inner antennae of PS2, CP43 and CP47, respectively (Bricker 1990, Groot *et al.* 1995). The intensity of fluorescence from the reaction centre of PS2 at 683 nm is lower than that of LHC2, CP43,

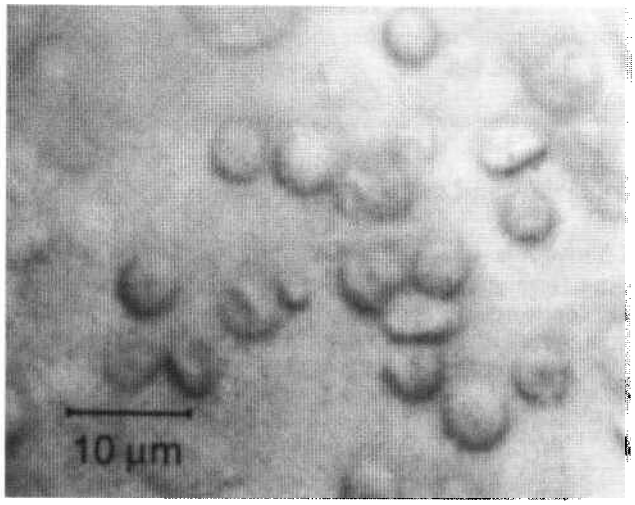

Fig. 1. Image of isolated chloroplasts observed by optical microscope in a reflection mode and detected by a CCD camera.

or CP47, and therefore not detectable in the spectrum. On the other hand, PS1 emits the fluorescence at 77 K only; the signal is located in the region of 715–745 nm with the maximum at 735 nm. This single, rather broad band is composed of PS1 reaction centre with its maximum of fluorescence emission at 725 nm and the light-harvesting complex of PS1 peaking at 735 nm (Knoetzel *et al.* 1992).

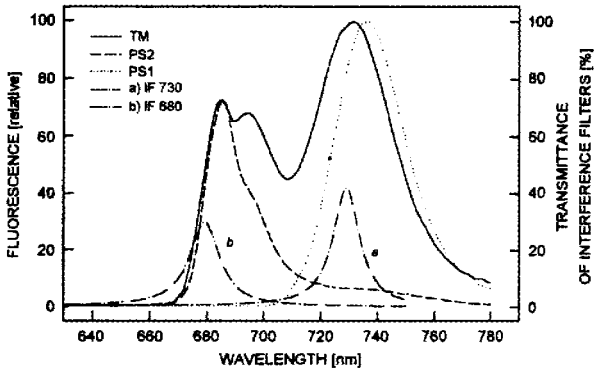

Fig. 2. 77 K fluorescence emission spectra of thylakoid membrane (*solid line*), photosystem 2 particles (*dashed line*), and photosystem 1 particles (*dotted line*). The fluorescence was excited at 470 nm, chlorophyll concentration was 10 g m^{-3}. Spectra of both photosystems are normalised at their respective maxima in the thylakoid membrane spectrum. The *dashed-dotted lines* show the transmittance of interference filters *IF 730* (*a*) and *IF 680* (*b*).

When observed by the laser scanning microscope at 77 K, the fluorescence images of chloroplasts looked similar to those in Fig. 1. Again, the concentration was adjusted so that a single layer of well-separated particles was achieved. One selected chloroplast scanned in detail is shown in Fig. 3. A krypton laser line at 647.6 nm was used to excite the Chl *b* of LHC2, which transfers the energy to PS2. To establish the *in vivo* localisation of both photosystems, we detected the fluorescence emission of the intact chloroplast using two filter sets. The first set was selected to detect only the emission of PS2 at 680 nm, containing a 3 mm cut-off filter *RG 665* (*Schott*, Germany) and an interference filter *IF 680* (*JVO*, Japan). The second set was selected to detect the emission of PS1 at 730 nm, containing a 3 mm cut-off filter *RG 715* (*Schott*) and an interference filter *IF 730* (*JVO*). The spectral characteristics of both interference filters are ploted in Fig. 2.

PS1 and PS2 are located in different regions of chloroplast and exhibit different fluorescence emission spectra. Therefore, we were able to observe domains of thylakoid membrane with predominant location of either of these photosystems.

Since chloroplasts of mature leaves have numbers of grana regions overlapping each other when viewing from the top, such chloroplasts generally emit the fluorescence mainly below 700 nm all over their surface. For that reason, we used chloroplasts isolated from young leaves, which have much lower numbers of grana stacks. The top views of these chloroplasts can reveal separated regions formed by stroma lamellae and regions of grana membranes.

Fig. 3 is a processed composition of two overlaid fluorescence emission micro-images of a single chloroplast detected using different filter sets. The first image was detected at 680 nm and superimposed upon the second image that was obtained by subtracting the fluorescence image detected at 680 nm from the image detected at 730 nm. The mathematical operations of subtraction and division supplied similar results; the subtraction figure was used for presentation since the division resulted in the high noise in the marginal regions due to the small divisors.

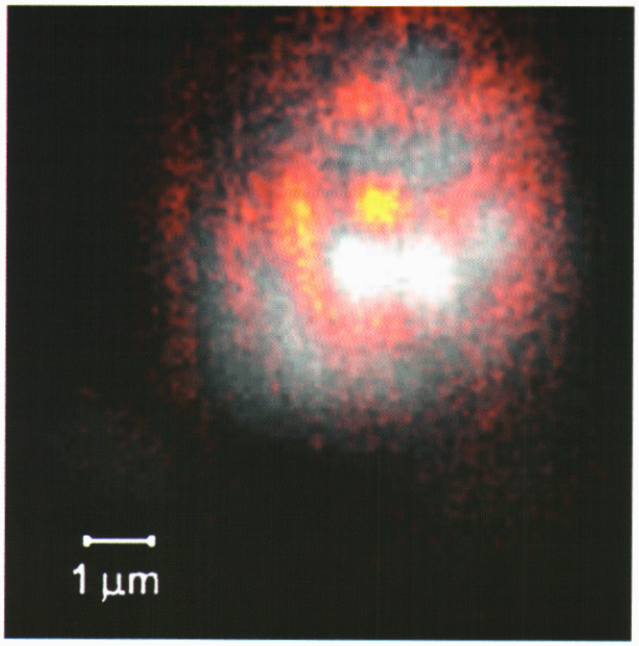

Fig. 3. Fluorescence emission image of a single chloroplast measured at 77 K. The grey scale shows the grana regions; the red-yellow scale represents the stroma lamellae regions. The image was processed by overlaying two images detected at different wavelengths. The first image of grana regions was obtained by detecting the fluorescence emission of photosystem 2 using a 680 nm interference filter (*IF 680*). The second image of stroma lamellae was obtained by subtracting the first image from the image detected by using a 730 nm interference filter, which represents mainly the fluorescence emission of photosystem 1.

The grana regions, where PS2 is mainly located, are represented by grey colour. The structures have mostly circular shape about 0.8 µm in diameter, which is slightly more than what is usually observed by electron microscopy. This may be caused by the fact that due to the size of the chloroplast, parts of the image in the axial direction could be slightly out of focus. Overlapping of

neighbouring grana may also make the spots look bigger. However, the shape strongly supports the previously reported circular-helical shape deduced from electron micrographs (Brangeon and Mustárdy 1979, Mustárdy 1996). The intensity of the fluorescence emission signal of grana differs within the chloroplast. It is probably due to the overlaying of several grana regions over each other in the middle of the chloroplast while in the chloroplast margins there is not enough space for more than one granum as it can be deduced from electron micrographs of spinach chloroplast (Murakami 1992). The maximum fluorescence intensity-to-background ratio in the grana regions is about 8.

The stroma lamellae regions, with predominant location of PS1, are represented by the scale of red-yellow colour. While the fluorescence signal excited by 647.6 nm krypton line in Chl *b* of LHC2 and detected at 680 nm is only due to the fluorescence of PS2, the fluorescence signal detected above 715 nm is composed of several contributions. The main fluorescence emitting species at around 730 nm is PS1. It can be excited by the 647.6 nm krypton line either *via* the Chl *a* coupled to PS1 or by energy transfer from LHC2 to PS1. However, PS2 emits fluorescence also at 730 nm and the laser beam of the microscope can pass through several layers of thylakoid membrane when penetrating the chloroplast and excites the overlapping grana and stroma lamellae regions contributing to the emission beyond 715 nm. The stroma lamellae fill the whole space of the chloroplast among the white spots of grana. The size of these individual stroma lamellae varies from 0.5 to 1 μm. Maximum fluorescence intensity of the stroma lamellae regions was about three times less then what was detected in grana regions. This does not necessarily mean that there is less of PS1 because of different fluorescence intensity of both photosystems when excited in Chl *b*. Different transmittance of the two filter sets also influences the intensity of detected fluorescence.

In conclusion, we succeeded for the first time in detecting and resolving the inner structure of intact chloroplasts of higher plants. The structure is the closest to *in vivo* state ever observed. The observed structure well corresponds to the earlier observations by electron microscopy (Brangeon and Mustárdy 1979, Murakami 1992, Mustárdy 1996). The presented results show a wide range of new possibilities in microscopic spectroscopy on photosynthetic systems both at low and room temperatures.

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