The Variation of the Electrochromic Difference Spectrum at Various Stages of the Chloroplast Development +

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The flash-induced difference spectrum in the range of 450–550 nm of protochloroplasts isolated from pea-leaves greened under intermittent illumination (2 min light, 98 min dark) was measured and compared with that of fully developed chloroplasts from pea leaves. Because of the sensitivity of the decay o fthe absorption changes to the ionophore valinomycin they were shown to mainly be due to an electrochromic bandshift of the membrane pigments (chlorophylls-a, -b and carotenoids). The differences in the shape and the amplitude between both spectra are consistently explained within the framework of a recent hypothesis supposed by Sewe and Reich (Z. Naturforsch. **33 c**, 161–171 (1978)) by the lack of chlorophyll-b in the protochloroplasts. It is concluded, that the transformation of the protochloroplasts into chloroplasts which is accompanied by the incorporation of the light harvesting complex and the formation of grana stacks does not seriously change the orientation of the field indicating pigments within the membrane with respect of the polarity of the light induced vectorial electron transport.

Introduction

The primary photoprocesses at the reaction centers of photosynthesizing organisms lead to the formation of an electric potential difference across the thylakoid or chromatophore membrane. The pigments embedded into these membranes undergo an electrochromic bandshift and give rise to field indicating absorption changes (for review s. ref. [1 – 3]). As the electrochromic absorption changes can kinetically be labelled by their sensitivity to ionophores which enhance the membrane permeability selectively for specific ions, the difference spectrum is easily separable from the absorption changes caused by other reactions (*e. g.* redox reactions). The electrochromic difference spectrum in green plants was found to be characterized by a negative peak at 480 nm and a pronounced positive peak at around 518 nm, together with smaller changes in the red and blue region [4]. It was inferred that the electrochromic effect is mainly due to the bandshift of the bulk pigments [1 – 3]. Furthermore, the absorption change at 518 nm was shown to be strongly dependent on the chlorophyll-b-content [5] and of the size of the photosynthetic unit [6]. On the basis of *in vitro* measurements a molecular model was proposed, which explains the electrochromic 518 nm absorption change as to mainly be due to the bandshift of a chlorophyll-b-lutein-complex, whereas the 480 nm peak is predominantly caused by chlorophyll-b [7]. The carotenoid moiety of the complex is oriented in such a way that the difference of the permanent dipole moments between the excited and the ground state of the complexed lutein has a nonzero component in the direction of the light induced electrical field across the thylakoid membrane [7].

During the ontogenesis of the chloroplasts from protochloroplasts chlorophyll-b was synthesized only after the development of the photosynthetic electron transport [8]. Accordingly, changes of the amplitude and the difference spectrum of the electrochromic absorption changes are anticipated to occur during chloroplast development. Recently, Akoyunoglou and Tsimilli-Michael [9] reported the existence of negative light induced 515 nm absorption changes in bean leaves greened under periodic light. After a postillumination of several hours the 515 nm absorption change became positive, *i. e.* of the same sign as in normal chloroplasts was observed. This might indicate drastic changes in the structural organization of the membrane during chloroplast development, *i. e.* the orientation between the Chl-b-lutein-complexes and the direction of the light-induced electric field might be changed. However, the

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interpretation of these results appear to be rather complex due to the possible interference with scattering effects and other types of absorption changes.

In order to clarify the process of the formation of the field indicating chlorophyll-b-lutein-complex and its orientation within the membrane, flash induced absorption changes and their sensitivity to ionophores have been investigated in protoplasts isolated from pea leaves. The results obtained do not support the occurance of a negative 515 nm absorption change. The data can consistently be explained within the framework of the hypothesis of Sewe and Reich [7] by the increased incorporation of chlorophyll-b and complex formation with lutein without drastic reorientation effects.

Materials and Methods

Protochloroplasts and chloroplasts were prepared under dim green light according to the method of Winget *et al.* [10]. Additionally, 10 mM ascorbate were present during grinding. Etiolated pea leaves *(Pisum sativum)* were used as the starting material. The growth conditions were the same as those described in ref. [11]. Five-days-old leaves were exposed to 28 light dark cycles of 2 min light and 98 min dark. The protochloroplasts were isolated from leaves grown under these conditions. For the full development of the chloroplasts 5-days old leaves were illuminated 32 hours under continuous light (8 hours light, 16 hours dark).

The chlorophyll content was determined after aceton extraction by the application of the extinction coefficient of McKinney [12]. The reaction mixture contained: Protochloroplasts or chloroplasts (10 μM chlorophyll), 100 μM benzylviologen, 10 mM KCl, 2 mM $MgCl_2$ and 20 mM morpholinoethanesulfonat-buffer, pH = 6.5. Reaction medium for the phosphorylation experiments: 10 μM chlorophyll, 100 μM benzylviologen, 10 mM KCl, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 20 mM Tricine-NaOH, pH = 8.0 and 0.3 mM ADP (as indicated in the figure). The measurements of the absorption changes were performed by a repetitive flash spectrophotometer as described in ref. [13]. Excitation with short flashes ($\lambda \geqq 610$ nm, $\tau \cong 20$ μs) at a repetition rate of 0.5 Hz. About 100 signals were stored in a Nicolet-averager, model 527. The electrical bandwidth ranged from 0 – 5 kHz, optical bandwidth 5 nm, optical pathlength 2 cm. The intensity of the measuring light beam was < 3 $\mu W/cm^2$.

The oxygen measurements were carried out by a repetitive flash-polarographic technique as described in ref. [14].

Results and Discussion

Flash-induced absorption changes at 524 nm in protochloroplasts and in normal chloroplasts are shown in Fig. 1. In order to confirm these absorption changes to be due to an electrochromic effect caused by the light induced electric potential difference across the membrane the sensitivity towards the ionophore valinomycin was tested. As is shown in Fig. 1 the 524 nm absorption change in protochloroplasts exhibits practically the same increase of the decay rate in the presence of valinomycin as normal chloroplasts, *i. e.* the absorption change is predominantly electrochromic.

In normal chloroplasts the decay of the 515 nm absorption change was found to become accelerated under phosphorylating conditions [15]. The data in Fig. 2 show, that also the decay of the 515 nm absorption change in protochloroplasts is similarly enhanced under phosphorylating conditions. Hence, in the protochloroplasts used for the present investi-

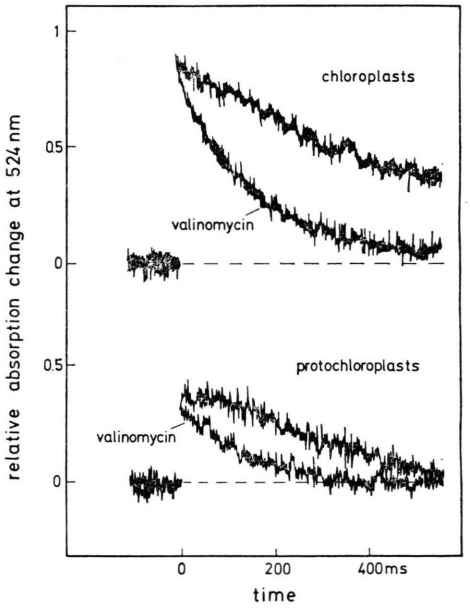

Fig. 1. Relative absorption change at 524 nm after excitation with single turnover flashes in chloroplasts and protochloroplasts. (Maximal absorption change at 520 nm $\Delta I/I = 1.6 \times 10^{-3}$). 64 signals were sampled from protochloroplasts, 16 from chloroplasts. Addition of 10^{-9} M valinomycin accelerates the decay of the absorption change in both cases by a factor 2 – 3. Details see Materials and Methods.

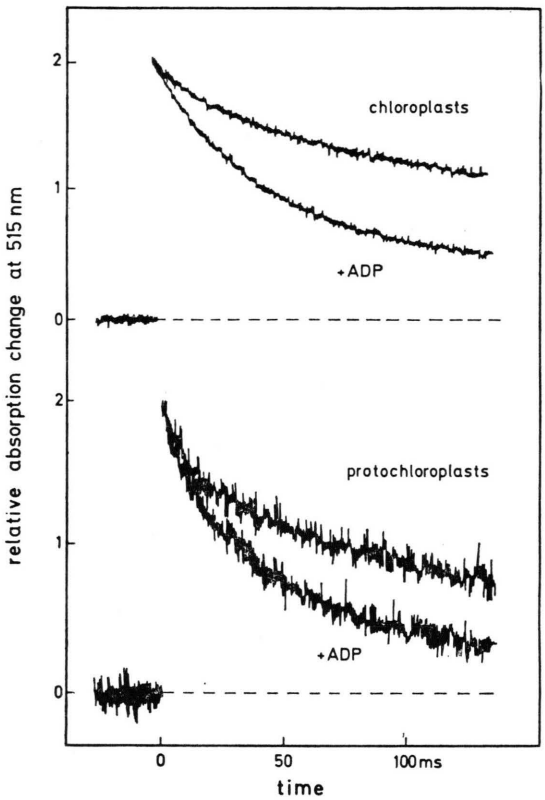

Fig. 2. Relative absorption change at 515 nm after excitation with a flash group under non-phosphorylating and under phosphorylating (+ADP) conditions in chloroplasts and protochloroplasts. 128 signals were sampled; 5 flashes per group, darktime withing the group 2 ms, between the flash groups 10 s.

gation the coupling between the ATPase and the transmembrane electrical field is practically the same as in normal chloroplasts. Furthermore, measurements of the average oxygen yield per flash under repetitive excitation conditions indicate that the watersplitting enzyme system Y is fully developed in the protochloroplasts. The size of the "photosynthetic unit" slightly varied in the protochloroplasts used for the present studies, it ranged from 50% – 100% of that found in the chloroplasts. In Fig. 3 the difference spectra in the range of 450 – 550 nm of normal chloroplasts and of protochloroplasts are depicted. Practically, the total amplitude of these absorption changes can be accelerated by addition of the ionophore valinomycin with exception of the absorption changes at 553 nm and 545 nm in protochloroplasts. In this case only 10% or 20%, respectively, can be accelerated, so that only 10% or 20% of the absorption change at these

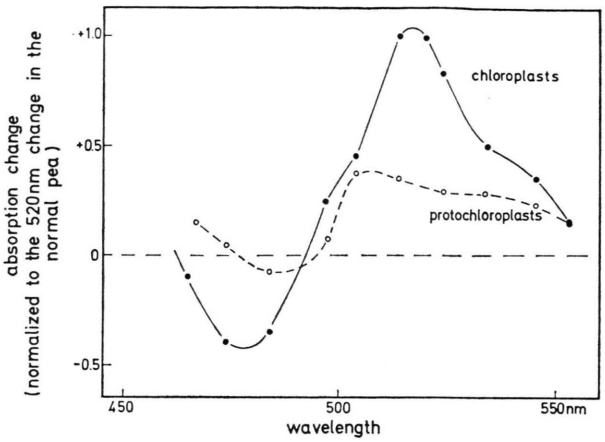

Fig. 3. Spectra of the flash-induced absorption changes from chloroplasts and protochloroplasts normalized to the absorption change at 520 nm ($\Delta I/I = 1.6 \times 10^{-3}$).

wavelengths are electrochromic. The comparison of the difference spectra reveals 3 significant phenomena:

a) Based on the total chlorophyll content the amplitude at 515 nm in the protochloroplasts is at least 3 times smaller than in normal chloroplasts.

b) In protochloroplasts the negative peak at 480 nm is much less pronounced compared with the positive peak than the corresponding peak to peak ratio in normal chloroplasts.

c) The maximum of the positive peak in protochloroplasts is blue-shifted by about 10 nm compared with the maximum observed in normal chloroplasts.

If one supposes that the area per electron transport chain and the thickness of the "impermeable" membrane core do not significantly change during the protochloroplast → chloroplast-transformation, then nearly the same electric field is established across the membrane (the dielectric constant is assumed to be practically invariant to the transformation). Accordingly, the reduced amplitude of the electrochromic absorption changes can be explained either by a reduced number of field indicating pigments (and/or pigment complexes) or by a decrease of the component of the difference of the permanent dipoles in the excited and ground state, respectively, in the direction of the light induced electric field due to orientational changes during the chloroplast formation. The rather small 480 nm absorption change can easily be explained by a drastically

reduced chlorophyll-b-content in the protochloroplasts [8]. As a consequence of the chlorophyll-b-deficiency also the number of the chlorophyll-b-lutein complexes should drastically be reduced, so that the decrease of the amplitude at 515 nm is inferred to mainly be due to a significantly reduced number of the field indicating pigment complexes rather than by a structural effect. This assumption is consistent with the 10 nm hypsochromic shift of the maximum of the positive green peak, because according to *in vitro* measurements [7] the maximum of the difference spectrum of the linear electrochromic effect of chlorophyll-a-lutein complexes is about 6 nm blue-shifted compared with that of the chlorophyll-b-lutein complexes. Accordingly, within the framework of the above mentioned *in vitro* data the present results can consistently be explained by the lack of chlorophyll-b in the membranes of the protochloroplasts. According to the data of Sewe [16] a more pronounced negative peak in the range around 490 nm would be expected for the electrochromic difference spectrum of the protochloroplasts, if one assumes, that mainly chlorophyll-a-lutein-complexes are the field indicating pigments in the range of 470 – 540 nm (chlorophyll-a alone should not significantly contribute to the spectrum). This difference as well as the small blue shift compared with the *in vitro* spectra might be explained by environmental effects.

On the basis of the present results the changes in the difference spectrum of the electrochromic absorption changes during the protochloroplast → chloroplast-transition can simply be understood by the incorporation of chlorophyll-b into the thylakoid membrane and the formation of chlorophyll-b-lutein-complexes.

Despite of the fact, that quantitative conclusions cannot be drawn from the present results, it is furthermore concluded, that there do not occur gross structural changes with respect to the orientation of the field indicating pigments, even during the incorporation of the light harvesting protein and the formation of grana stacks. Our results do not confirm the occurance of negative absorption changes around 515 nm [9]. The data of ref. [9] seemed to be caused by an interference with absorption changes, which are not electrochromic (*e. g.* scattering effects) and/or the reference to 540 nm, where other absorption changes occur (data not shown).

After the completion of this study we became aware of the results of J. Farineau, obtained on etioplasts of maize (mesophyll cells), which are in close agreement with our data [17].

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