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Participation of Free Radicals in Photoreduction of Protochlorophyllide to Chlorophyllide in an Artificial Pigment–Protein Complex

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Abstract—The primary stages of protochlorophyllide phototransformation in an artificially formed complex containing heterologously expressed photoenzyme protochlorophyllide-oxidoreductase (POR), protochlorophyllide, and NADPH were investigated by optical and ESR spectroscopy. An ESR signal (g = 2.002; H = 1 mT) appeared after illumination of the complex with intense white light at 77 K. The ESR signal appeared with simultaneous quenching of the initial protochlorophyllide fluorescence, this being due to the formation of a primary non-fluorescent intermediate. The ESR signal disappeared on raising the temperature to 253 K, and a new fluorescence maximum at 695 nm belonging to chlorophyllide simultaneously appeared. The data show that the mechanism of protochlorophyllide photoreduction in the complex is practically identical to the *in vivo* mechanism: this includes the formation of a short-lived non-fluorescent free radical that is transformed into chlorophyllide in a dark reaction.

Key words: protochlorophyllide, protochlorophyllide-oxidoreductase, non-fluorescence intermediate, ESR signal

Chlorophyllide is formed from its precursor protochlorophyllide (Pchlde) in etiolated leaves of higher plants in a special native complex that contains photoenzyme protochlorophyllide-oxidoreductase (POR), Pchlde, and the hydrogen donor NADPH. POR, an enzyme of the alcohol dehydrogenase family, was isolated, investigated, and described in the works of Griffiths and collaborators [1-5]. Its molecular mass is 36 kD [6]. POR is the catalyst for the photochemical transformation of Pchlde into chlorophyllide. The photoreduction of Pchlde includes several short-lived intermediates [7-18].

We previously studied Pchlde photoreduction in intact etiolated leaves using optical and ESR spectroscopy [13, 14]. ESR signals were found to be photoinduced at 77 K. These signals are due to the formation of two products of the primary stages of Pchlde photoreduction. The first stage was revealed by the quenching of the initial Pchlde fluorescence without the appearance of any new absorption maxima. No change in the absorption spectrum was observed. This stage was studied at low temperatures (4.2-77 K) [11, 12] and at room temperature using nano- and picosecond spectroscopy [15-18]. It is known that quenching of fluorescence without change in the absorption spectrum is possible when an unstable charge-transfer complex is transformed into a more stable complex [19]. The product of the first reaction (called in our work intermediate R) is subject to fast back transformation into Pchlde. The forward reaction led to the formation (from intermediate R) of the earlier found intermediate X690—a second non-fluorescent product with an absorption band at 690 nm. This reaction was observed at 70-140 K [7-11]. The ESR signal appeared simultaneously with the formation of the R and X690 intermediates [13, 14]. The signal disappeared when X690 transformed into chlorophyllide. These data suggest that in the initial stages of Pchlde photoreduction *in vivo* at least two paramagnetic non-fluorescent intermediates are formed. The connection of the ESR signal with intermediates of Pchlde photoreduction was confirmed in *in vitro* experiments. Pchlde photoreduction using different hydrogen donors in solution at a low temperature (143 K) produced a singlet ESR signal that was

Abbreviations: Pchlde) protochlorophyllide; Chlde) chlorophyllide; POR) protochlorophyllide-oxidoreductase; MBP) maltose binding protein; NFI) non-fluorescent intermediate.

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suggested to be due to the pigment anion radical [20]. The ESR signal appeared when protochlorophyll solution was illuminated at 77 K even without special hydrogen donors [13, 14]. Simultaneously, protochlorophyll fluorescence was quenched. The quenching of monomeric protochlorophyll (low pigment concentration) fluorescence was completely reversible on raising the temperature. An intermediate analogous to intermediate R *in vivo* was apparently formed in this case. The process stopped at this stage and was completely reversible. Solvent molecules apparently served as the electron donor.

We thought it would be of interest to investigate using ESR spectroscopy the mechanism of the intermediate stages of Pchlde photoreduction in a model system more similar to the native *in vivo* complex of Pchlde. The isolation and purification of POR [1-5] allowed study of the process of chlorophyllide formation under artificial conditions using the components of the native complex. However, the first attempt to observe an ESR signal in an artificial complex containing POR, Pchlde, and NADPH illuminated at 77 K was not successful [21]. Recently, a weak ESR signal was observed in analogous complexes illuminated at 223 K [22].

Here we report on an investigation of Pchlde photoreduction *in vitro* in the artificial complex using optical and ESR spectroscopy with lower temperature of irradiation (77 K) and higher pigment concentrations, which apparently promote the formation of the main active Pchlde form.

MATERIALS AND METHODS

An artificial pigment–protein complex was prepared using MBP-POR fusion protein. MBP-POR was obtained by the overexpression of the pea POR gene into *Escherichia coli* [21]. Pchlde was isolated from mutant *Rhodobacter sphaeroides* V3 as described earlier [23]. NADPH was obtained from Sigma (USA).

Sample preparation. A triple complex containing Pchlde, MBP-POR, and NADPH was prepared as follows: 0.025 ml of concentrated ($6\cdot10^{-2}$ M) methanol solution of Pchlde and 0.025 ml of NADPH ($4\cdot10^{-3}$ M) in HEPES (50 mM, pH 7.4) were added to 0.5 ml of MBP-POR. After incubation in the dark at 293 K (15-60 min), the complex was ready for phototransformation. Samples (0.25 ml) of the prepared mixture were placed in a special quartz ampoule for measurements of fluorescence and ESR spectra.

Irradiation of samples. To investigate the primary stages of Pchlde phototransformation, samples were illuminated at 77 K with white light from a 500 W xenon lamp. The light intensity at the surface of the samples was $5\cdot10^3$ W/m^2.

Spectroscopy. Fluorescence emission spectra were recorded at 77 K using an apparatus including an MDR-2 wide-aperture monochromator and a FEU-83 photomultiplier [24]. Absorption spectra were measured using an SF-18 recording spectrophotometer equipped with an integrating sphere. ESR spectra were recorded using an RE-1307 radiospectrometer (0.5 mW). A thermostatic device was used to vary the temperature in the resonator from 77 to 293 K.

RESULTS

A concentrated methanol solution of Pchlde ($6\cdot10^{-2}$ M) was used to form the active complex. The high pigment concentration allowed the formation of the triple complex (Pchlde, POR, NADPH) containing an active Pchlde form with spectral characteristics very close to those of the active Pchlde form *in vivo*: the main absorption band of the complex was located at 648 nm (Fig. 1), and the corresponding fluorescence band was observed at 651 nm (Fig. 2). The weak monochromatic light in the SF-18 spectrophotometer did not cause Pchlde phototransformation (controls were performed at 77 K and at room temperature). Unfortunately, the high pigment concentration led to the formation of an aggregate with fluorescence maximum at 708 nm. The amount of this aggregate was not large because its maximum was not observed in the absorption spectra. The high intensity of the fluorescence band at 708 nm indicated intensive energy migration from the main Pchlde form (A648, F651) to the aggregate of the pigment.

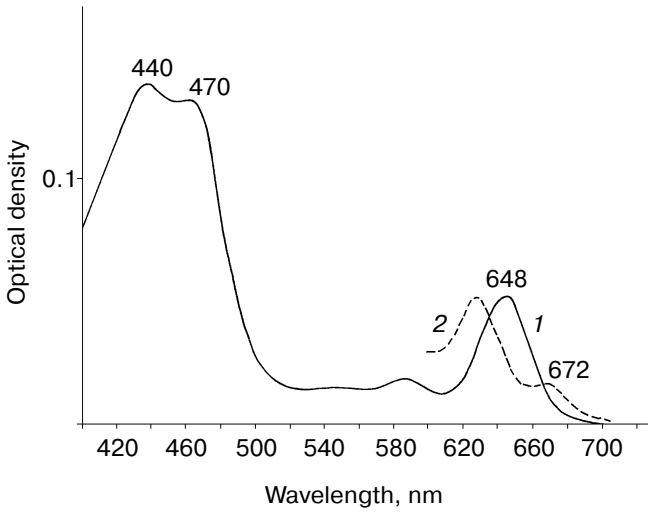

Fig. 1. Absorption spectra: *1*) non-illuminated complex of Pchlde, MBP-POR, and NADPH; *2*) methanol extract from sample *1* after it had been illuminated at 77 K and heated to 253 K.

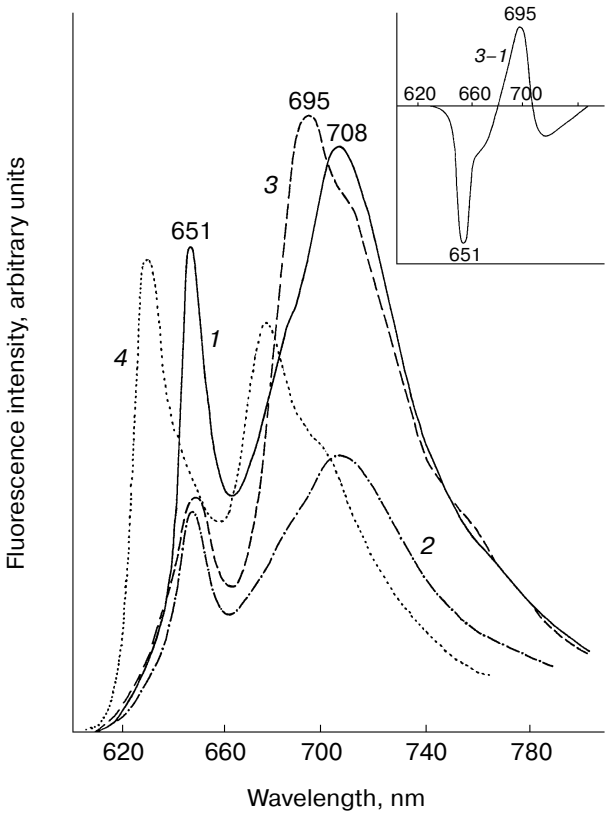

Fig. 2. Primary stages of Pchlde photoreduction in the complex containing Pchlde, MBP-POR, and NADPH. Low temperature (77 K) fluorescence spectra of the complex: *1*) before illumination; *2*) after 10 min illumination with white light ($5 \cdot 10^3$ W/m^2) at 77 K; *3*) after increasing the temperature of the illuminated sample to 253 K; *4*) spectrum of methanol extract from the sample that had been illuminated at 77 K and heated to 253 K. Inset, difference spectrum (curve *3* minus curve *1*).

Illumination of the sample with white light ($5 \cdot 10^3$ W/m^2) at 77 K led to a marked (60%) quenching of both fluorescence bands (Fig. 2). No new bands appeared. It is known that the photoinduced decrease in the fluorescence maximum at 655 nm *in vivo* is accompanied by the formation of a non-fluorescent intermediate [7-14]. The parallel decrease in the band at 708 nm is apparently the result of a decrease in energy migration from Pchlde 651 after its transformation into the NFI.

In the ESR spectra (Fig. 3) of the non-illuminated samples, no dark signal at 77 K was observed. After illumination of samples with intense light at 77 K a singlet ESR signal with g-factor 2.002 characteristic of the free electron and width of 1 mT appeared. Therefore, the appearance of this ESR signal correlates with fluorescence quenching, i.e., the a non-fluorescent paramagnetic intermediate was formed (the same behavior occurred *in vivo*). After increasing the temperature of the illuminated sample to 253 K, the ESR signal disappeared and a new maximum at 695 nm appeared in the fluorescence spectrum. The difference spectrum (Fig. 2, inset) shows that a new pigment form with maximum at 695 nm is formed from Pchlde with fluorescence maximum at 651 nm. In the absorption and fluorescence spectra of a methanol extract from a sample illuminated at 77 K and heated to 253 K, new bands at 672 nm (Fig. 1) and 682 nm (Fig. 2), respectively, were found. These bands belong to chlorophyllide. Consequently, the fluorescence band at 695 nm that was found in the spectrum of the complex after illumination at 77 K and increase in temperature is due to the primary chlorophyllide form.

Thus, in the artificial active complex containing Pchlde, POR, and NADPH the photoreduction of Pchlde apparently proceeded in the same way as in intact etiolated leaves. This process includes the formation of a short-lived non-fluorescent free radical intermediate that is transformed into chlorophyllide in a dark reaction.

When NADH was used as a component of the artificial complex instead of NADPH, the main maxima of the low temperature fluorescence spectrum were located at 644 and 703 nm. Fifty percent fluorescence quenching and a weak ESR signal (Fig. 3) were observed after illumination of the sample with NADH at 77 K. However, after increasing the temperature to 253 K, only a back reaction took place: an increase in Pchlde fluorescence and no new fluorescence bands were observed. The same picture was observed when the

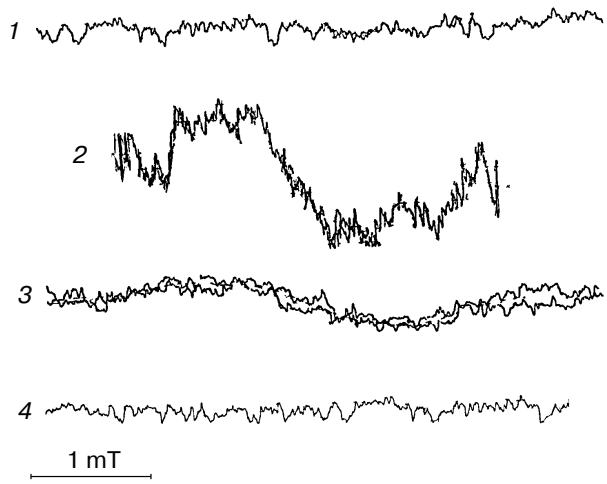

Fig. 3. ESR spectra of the complex containing Pchlde, MBP-POR, and NADPH measured at 77 K (curves *1* and *2*) and at 153 K (curves *3* and *4*). Curve *1* is the spectrum of the non-illuminated complex of POR, Pchlde, and NADPH; curves *2* and *3* are spectra of the same complex after 10 min of illumination with $5 \cdot 10^3$ W/m^2 white light at 77 K; curve *4* is the spectrum of the illuminated complex after increasing the temperature to 253 K.

denatured (5 min heating at 363 K) POR was used for the formation of the complex. The low temperature fluorescence spectrum of this complex was very similar to the spectrum of concentrated Pchlde solution: the main maxima were located at 641 and 701 nm. However, 35% fluorescence quenching was observed after illumination of the sample with intense white light at 77 K. After increasing the temperature to 253 K, only an increase in the Pchlde fluorescence was also observed. Consequently, the primary NFI that was revealed by the quenching of the initial Pchlde fluorescence was formed even in the absence of the natural electron donor NADPH or active enzyme. However, the transformation of the NFI into chlorophyllide did not occur in this case; only the back reaction was observed. These results are consistent with our earlier data [13, 14] that were obtained with a solution of monomeric protochlorophyll (see the introductory section). Is seems possible to explain these facts by the formation of the primary non-fluorescent intermediate R that is reversible on increasing the temperature. Further transformation of this intermediate into chlorophyllide without a specific hydrogen donor or enzyme did not occur.

DISCUSSION

Therefore, the results of the present study indicate that the photoreduction of Pchlde *in vitro* (in a complex containing Pchlde, NADPH, and MBP-POR) occurs in two stages:

$$\text{Pchlde F651} \xrightarrow{h\nu} \text{NFI (free radical)} \xrightarrow{\text{dark}} \text{Chlde F695}.$$

The first stage is observed as the quenching of the initial Pchlde fluorescence (formation of a non-fluorescent intermediate). A weak ESR signal simultaneously appears. The parameters of ESR signal ($g = 1.002$, $\Delta H = 1$ mT) show free radical formation at this stage. The second stage is the dark temperature-dependent reaction of chlorophyllide formation from the NFI.

Comparison of these data with the results obtained with intact etiolated leaves [13, 14] shows that the mechanism of the primary reaction of Pchlde photoreduction in the artificial complexes is very similar to the mechanism of the primary reactions *in vivo*.

Somewhat different results were obtained in the above-mentioned work [22]. The authors concluded that a weak ESR signal that was seen after illumination of the artificial complex at 223 K was due to a pigment form having a fluorescence band at 682 nm. Comparing our results with those reported in [22], it should be noted that the fluorescent properties of Pchlde in the complexes, demonstrated in this work, were significantly different from the spectral properties of the main active Pchlde form in intact etiolated leaves (F655-657): the maximum of the active protochlorophyllide was located at 645 nm. It is known [25] that the fluorescence lifetime of Pchlde645 *in vivo* is about 10 times longer than the fluorescence lifetime of Pchlde655. Moreover, the photochemical activity of Pchlde645 *in vivo* is more dependent on temperature than the photochemical activity of Pchlde655 [26]. Consequently, the mechanisms of photoreduction of these two forms must be different. Therefore, it is possible that the differences in the identification of paramagnetic intermediates in our study and in [22] due to differences in the mechanisms of photoreduction of different Pchlde forms. On the other hand, the photoinduced ESR signal was observed in work [22] at a sufficiently high temperature (223 K), that is, under conditions which do not promote the stabilization of the primary non-fluorescent intermediate. In our experiments with intact etiolated leaves [13, 14], a weak ESR signal was still observed at 223 K with simultaneous formation of the primary fluorescent chlorophyllide forms. Apparently, at 223 K (in work [22]) the process was also at the stage of transformation of the non-fluorescent paramagnetic intermediate into the primary chlorophyllide forms. In this case, both intermediates, the NFI (the free radical giving rise to the ESR signal) and the primary fluorescent chlorophyllide form could be seen.

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