REDUCTION OF PHEOPHYTIN IN THE PRIMARY LIGHT REACTION OF PHOTOSYSTEM II

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1. Introduction

The photochemical reduction of pheophytin and bacteriopheophytin has been shown in vitro [1–3]. In reaction centers of photosynthetic bacteria bacteriopheophytin *a* [4–9] and bacteriopheophytin *b* [10, 11] act as an intermediary electron carrier between bacteriochlorophyll dimer and the 'primary' electron acceptor, a complex of ubiquinone and Fe. When the ubiquinone is in the reduced form the photoaccumulation of reduced bacteriopheophytin can be observed [5–11]. In various species of green plants 1.5–2.3 molecules of pheophytin have been found per 100 molecules of chlorophyll [12]. In photosystem II of green plants the photoreduction of the primary electron acceptor, Q (plastoquinone), is accompanied by a blue shift of absorption bands at 545 nm and 685 nm [13,14] which can belong to a bound or aggregated form of pheophytin in reaction centers of photosystem II [14]. The photoreduction of pheophytin may be observed in photosystem II preparations from pea chloroplasts at 20°C [15]. In this work a reversible reduction of pheophytin in the primary light reaction of photosystem II in pea subchloroplast particles at redox potentials (E_h) from −50 mV to −550 mV (when Q is in the reduced form) is demonstrated. This photoreaction is observed at −170°C as well as at 20°C and is accompanied by a 2–3 fold decrease in the chlorophyll fluorescence yield.

2. Materials and methods

The 'heavy' chloroplast fragments, enriched in photosystem II, were isolated when treating pea chloroplasts with digitonin (0.4%) and Triton X-100 (0.1%) followed by fractional centrifugation [16]. The fraction precipitated for 45 min at 20 000 × *g* and designated as 'DT-20 fragments' [16] was used. The chlorophyll–protein complexes of photosystems I and II and the 'accessory' complex were prepared using the chromatography on DEAE-cellulose of pea chloroplasts treated with 3% Triton X-100 [17]. The absorbance changes (ΔA) and the changes in the chlorophyll fluorescence yield (ΔF), induced by continuous actinic light, were measured with the phosphoroscopic technique described earlier [7,15]. The measurements were made in a 10 mm cuvette, in which the E_h value of the medium was registered under anaerobic conditions [6,7].

3. Results

In the DT-20 fragments at the medium E_h of +400 mV the actinic light induces a reversible increase, related to the photoreduction of Q [18], in the chlorophyll fluorescence yield (fig.1). Under these conditions the light-induced ΔA observed (fig.1) are similar to the positive ΔF in their kinetics, according to

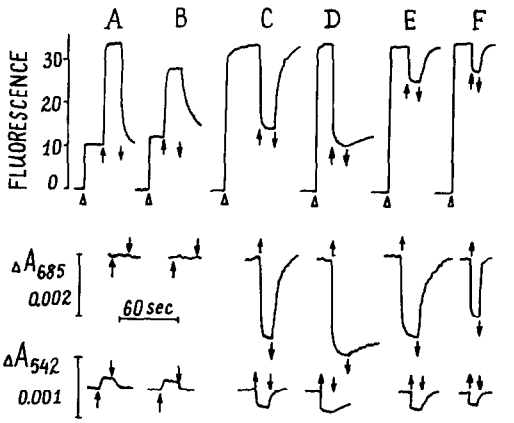

Fig.1. Kinetics of the light-induced fluorescence changes (ΔF) and of the absorbance changes (ΔA) at 685 nm and 542 nm of pea photosystem II preparations. (A–D) DT-20 fragments suspended in 20 mM Tris buffer, pH 8.5 (a chlorophyll concentration 20 μg/ml), at 20°C. (A) Without additions. (B) In the presence of 5 μM ferricyanide and 1 μM DCMU at E_h +400 ∓ 10 mV. (C,D) In the presence of 1 μM indigodisulfonate, 0.5 μM methylviologen and 0.2–2.0 mg/ml dithionite: (C) at E_h −200 ∓ 30 mV, (D) at E_h −400 ∓ 20 mV. (E) The chlorophyl–protein complex of photosystem II suspended in the same buffer (a chlorophyll concentration 7 μg/ml) in the presence of 1 μM methylviologen and 2.0 mg/ml dithionite at E_h −490 ∓ 10 mV.at 20°C. (F) The film, obtained by drying the suspension of DT-20 fragments in the presence of 0.5 mg/ml dithionite and 1 μM DCMU under anaerobic conditions (absorbance of the film at 680 nm was 1.6), the measurements were carried out at −170°C. (Δ) Monochromatic light (480 nm, a half bandwidth of 10 nm, intensity of 7 ergs·cm^{-2}·s^{-1}), exciting the chlorophyll fluorescence (λ > 660 nm), on. (↑) Actinic light (λ > 620 nm, 1.9·10^5 ergs·cm^{-2}·s^{-1}), on; (↓) actinic light, off.

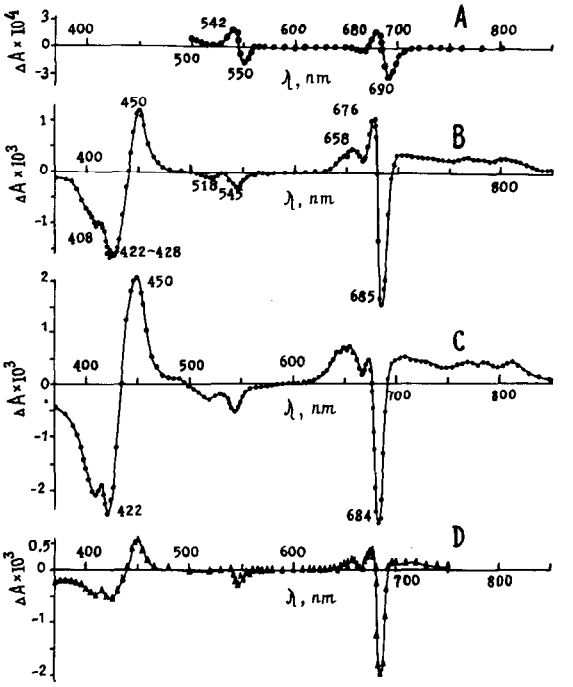

Fig.2. Difference absorption spectra ('light-minus dark') of pea photosystem II preparations. (A,B) Suspension of DT-20 fragments (a chlorophyll concentration 20 μg/ml) at E_h +400 mV (A) and at E_h −200 mV (B). (C) Suspension of the chlorophyll–protein complex of photosystem II (a chlorophyll concentration 7 μg/ml) at E_h −490 mV at 20°C. The additions as in fig.1. (D) The film, obtained by drying the suspension of DT-20 fragments in the presence of 0.5 mg/ml dithionite and 1 μM DCMU under anaerobic conditions (absorbance of the film at 680 nm was 1.6), the measurements were carried out at −170°C. To avoid ΔA related to the *P*-700 photooxidation the spectrum of fig.2A was measured in the presence of weak far-red background light (λ > 710 nm) [13,14] which oxidized *P*-700 and did not induce ΔA related to the Q photoreduction.

experiments with higher concentrations of fragments. The spectrum of these ΔA is characterized by negative bands at 550 nm and 690 nm and by two positive bands at 542 nm and 680 nm (fig.2A). It corresponds to the blue shift of absorption bands at 545 nm and 685 nm, which accompanies the photoreduction of Q [13,14].

At the E_h below −50 mV the chemical reduction of Q [18,19] is accompanied by the increase in the chlorophyll fluorescence yield up to its maximum level (fig.1). The actinic light induces a reversible decrease in the fluorescence almost to the level of 'dark' fluorescence registered at E_h +400 mV (fig.1). (The effect of the irreversible light-induced decrease in the chlorophyll fluorescence yield in both the pea chloroplasts and digitonin fragments of chloroplasts under reductive conditions has been described in detail earlier [19].) Under these conditions no ΔA, related to the photoreduction of Q, are observed but new reversible light-induced ΔA, which are similar in their kinetics to the negative ΔF, are detected (fig.1). The spectrum of these ΔA at the E_h of −200 mV is characterized by bleaching of the absorption bands at 545 nm and 685 nm as well as at 408 nm, 422–428 nm and 518 nm and by the development of a band at

676 nm and of broad bands at 450 nm, 658 nm and with $\lambda > 695$ nm (fig.2B). The dark decay of the light-induced ΔF and ΔA at 685 nm and 542 nm is slowed down when E_h is lowered from −200 mV to −400 mV (fig.1). This indicates the reductive nature of the photoprocess. At the E_h below −450 mV these spectral changes in DT-20 fragments were largely irreversible in the dark. However, in the chlorophyll–protein complex of photosystem II the light-induced ΔA, accompanied by the decrease in the fluorescence yield, were reversible in the dark up to E_h −550 mV. The spectrum of these ΔA at E_h −490 mV (fig.2C) is similar to the difference absorption spectrum of DT-20 fragments at E_h −200 mV (fig.2B). The value of the light-induced ΔA (per mg chlorophyll) in the complex of photosystem II is three times higher than that in DT-20 fragments (figs 1 and 2). In the 'accessory' complex and in the complex of photosystem I the photoreaction described was not observed. This can show its relation to photosystem II.

In DT-20 fragments the initial rate of the light-induced decrease in the chlorophyll fluorescence yield at E_h −450 mV $((\Delta F_i/\Delta_t)_{-450})$ and that of the light-induced increase in the fluorescence yield, following the Q photoreduction, at E_h +300 mV $((\Delta F_i/\Delta_t)_{+300})$ were just the same when the intensity of actinic light used at E_h −450 mV (I_{-450}) was 500 times higher than that used at E_h +300 mV (I_{+300}). When the rate of direct photoreaction is higher than that of dark reactions (that was observed in our experiments) $\Delta F_i/\Delta t$ is directly proportional to $\varphi . I . \Delta F_{max}$. Since the values of the maximum ΔF (ΔF_{max}) at E_h −450 mV and at E_h +300 mV are nearly the same (fig.1), the ratio of quantum yields of the photoreactions at −450 mV and at +300 mV $(\varphi_{-450}/\psi_{+300})$, when $(\Delta F_i/\Delta t)_{-450} = (\Delta F_i/\Delta t)_{+300}$, is reversely proportional to the ratio I_{-450}/I_{+300}, i.e. $\varphi_{-450}/\varphi_{+300} \approx 1/500$. Thus, if $\varphi_{+300} \approx 1$ [13] then $\varphi_{-450} \approx 0.002$.

The light-induced ΔA and ΔF of DT-20 fragments at the E_h below −50 mV were observed also in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). In 80% glycerol they were registered at −80°C as well as at 20°C. In the film obtained by drying the suspension of DT-20 fragments in the presence of dithionite and DCMU this photoreaction is also observed at −170°C (figs 1F and 2D) as well as at 20°C. The difference absorption spectrum (light minus dark) of this film at −170°C resembles that of the suspension of DT-20 fragments at 20°C (figs 2B and 2D) but the ΔA values of the film in the region 400–500 nm are relatively smaller (probably, due to more light scattering).

4. Discussion

The light-induced bleaching of the absorption bands near 420 nm and 680 nm and the appearance of bands near 450 nm, 660 nm and with $\lambda > 695$ nm at low redox potentials (fig.2) show that the photoreduction of either chlorophyll or pheophytin occurs in photosystem II preparations. (20-23/.) The bleaching of two small bands at 518 nm and 545 nm (fig.2) strongly indicates the photoreduction of pheophytin [20]. The development of the broad band near 450 nm (fig.2) is characteristic of the radical anion of pheophytin *a* [2]. In ether solution the absorption bands of pheophytin *a* are at 408 nm, 505 nm, 534 nm and 667 nm [20]. The shift of these bands in vivo to 422 nm, 518 nm, 545 nm and 685 nm, respectively, can be interpreted as an enviromental shift. However, the development of the narrow band at 676 nm can show that the pheophytin photoreduction results both in the blue shift of the chlorophyll absorption band at 680 nm and in the bleaching of the pheophytin band at 670 nm. Such an interpretation is similar to that suggested for the photoreduction of bacteriopheophytin in *Chromatium minutissimum* [5–7] and *Chr. vinosum* [8,9].

Pheophytin is probably reduced in the primary light reaction of photosystem II rather than in a photoreaction of antenna pigments, since this completely reversible photoprocess occurs at −170°C and is accompanied by a 2–3-fold decrease in the chlorophyll fluorescence yield of photosystem II when only approx. 0.1% of all the pigment molecules bleach. However, one cannot exclude completely the possibility that pheophytin, which can appear during the isolation of the chloroplast particles and which is not related directly to reaction centers of photosystem II, is also reduced under illumination at 20°C. This question is under study now.

The pheophytin photoreduction in photosystem II is similar to the earlier described [5–9] photoaccumulation of radical anion of bacteriopheophytin (Bph^-) in *Chromatium*. (This photoaccumulation

is a result of a fast ($\sim$ 1 μs) reaction of ferrocytochrome with a biradical of the reaction center, [P-890$^+$ Bph$^-$] [6,8,9].) Really, both the photoreactions occur only when the 'primary' electron acceptor (ubiquinone or plastoquinone) is in the reduced form. Both the photoreactions are accompanied by the decrease in the chlorophyll fluorescence yield and are observed at low temperatures but they have a low quantum yield. From this comparison, one can assume that the photoreduction of pheophytin (Ph) in photosystem II is also a result of electron transfer from a secondary electron donor of photosystem II to the biradical [P-680$^+$ Ph$^-$] which is formed in the primary photoact of photosystem II preceding the reduction of Q. (Here, P-680 is the primary electron donor of photosystem II [13,14, 21,22]. Then by analogy with the photoreaction in *Chromatium* [6,7], the fluorescence increase under the reduction of Q can be interpreted as the appearance of short-lived luminescence which is a result of the P-680 excitation in the charge recombination in the biradical [P-680$^+$ Ph$^-$]. The decrease in the luminescence, when pheophytin is photoreduced, can be due to the photoaccumulation of the inactive state of the reaction center, [P-680 Ph$^-$].

We wish to note that the formation of pheophytin during the procedure of photosystem II preparations should be studied to reveal the possible pheophytin photoreduction outside the reaction centers.

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References

[1] Krasnovsky, A. A. and Voinovskaya, K. K. (1951) Dokl. Acad. Nauk 81, 879–882.
[2] Evstigneev, V. B. and Gavrilova, V. A. (1954) Dokl. Acad. Nauk 96, 1201–1204.
[3] Pakshina, E. V. and Krasnovsky, A. A. (1974) Biophysica 19, 238–242.
[4] Rockley, M. G., Windzor, M. W., Cogdell, R. J. and Parson, W. W. (1975) Proc. Natl. Acad. Sci. USA 72, 2251–2255.
[5] Shuvalov, V. A., Klimov, V. V., Krakhmaleva, I. N., Moscalenko, A. A. and Krasnovsky, A. A. (1976) Dokl. Acad. Nauk 227, 984–987.
[6] Shuvalov, V. A. and Klimov, V. V. (1976) Biochim. Biophys. Acta 440, 587–599.
[7] Klimov, V. V., Shuvalov, V. A., Krakhmaleva, I. N., Karapetyan, N. V. and Krasnovsky, A. A. (1976) Biochimia 41, 1435–1441.
[8] Tiede, D. M., Prince, R. C. and Dutton, P. L. (1976) Biochim. Biophys. Acta 449, 447–467.
[9] Van Grondelle, R., Romijn, I. C. and Holmes, N. G. (1976) FEBS Lett. 72, 187–191.
[10] Shuvalov, V. A., Krakhmaleva, I. N. and Klimov, V. V. (1976) Biochim. Biophys. Acta 449, 597–601.
[11] Klimov, V. V., Shuvalov, V. A., Krakhmaleva, I. N., Klevanik, A. V. and Krasnovsky, A. A. (1977) Biochimia 42, 519–530.
[12] Krasnovsky, A. A. and Shaposhnikova, M. G. (1970) Physiol. Rastenij 17, 436–439.
[13] Van Gorkom, H. J., Tamminga, J. J. and Haveman, J. (1974) Biochim. Biophys. Acta 347, 417–438.
[14] Van Gorkom, H. J. (1974) Biochim. Biophys. Acta 347, 439–442.
[15] Klevanik, A. V., Klimov, V. V., Shuvalov, V. A. and Krasnovsky, A. A. (1977) Dokl. Acad. Nauk in press.
[16] Shutilova, N. I., Klimov, V. V., Shuvalov, V. A. and Kutjurin, V. M. (1975) Biophysica 20, 844–847.
[17] Shutilova, N. I. and Kutjurin, V. M. (1975) Biophysica 20, 246–249.
[18] Duysens, L. M. N. and Sweers, H. E. (1963) in: Studies on Microalgae and Photosynthetic Bacteria (Miyachi, S. ed) pp. 353–372, Univ. Tokyo Press, Tokyo.
[19] Karapetyan, N. V. and Klimov, V. V. (1973) Physiol. Rastenij 20, 545–553.
[20] Goedheer, J. C. (1966) in: The Chlorophylls (Vernon, L. P. and Seeley, G. R. eds) pp. 143–184, Academic Press, New York, London.
[21] Gläser, M., Wolf, Ch., Buchwald, H.-E. and Witt, H. T. (1974) FEBS Lett. 42, 81–85.
[22] Lozier, R. H. and Butler, W. L. (1974) Biochem. Biophys. Acta 333, 465–480.