THE PHOTOREDUCTION OF SPINACH FERREDOXIN IN THE PRESENCE OF PORPHYRIN AND AN ELECTRON DONOR

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Communicated October 20, 1967

It has previously been established that photosynthetic pyridine nucleotide reductase (PPNR)[1] or spinach ferredoxin (Fd)[2] is required for the reduction of pyridine nucleotides by illuminated chloroplasts. The reduction of Fd by illuminated chloroplasts has since been described by various workers.[3-5] In addition, it has been shown that Fd can be reduced by hydrogen and hydrogenase,[2] hydrosulfite, and hydrogen and platinum asbestos.[3] Furthermore, the photoreduction of Fd by illuminated chloroplasts with its subsequent oxidation in a dark reaction leading to the formation of reduced pyridine nucleotide has been demonstrated.[4] The NADP-reducing system of spinach chloroplasts is composed of two components, a ferredoxin and a flavoprotein enzyme referred to as ferredoxin-NADP reductase (spinach pyridine nucleotide transhydrogenase or spinach NADPH diaphorase). Evidence that both components are required for the reduction of pyridine nucleotides by illuminated chloroplasts, with Fd being reduced initially by the chloroplast, has been presented.[2, 6-10]

Krasnovsky[11] first demonstrated that chlorophyll photosensitized the reduction of a number of dyes in pyridine solution, with ascorbic acid as the electron donor. Thereafter, it was shown[12, 13] that chlorophylls dissolved in pyridine catalyzed a photoreduction of pyridine nucleotides by ascorbate. Vernon[14] reported that various porphyrins and porphyrin derivatives, as well as chlorophyll, catalyzed the photoreduction of NAD and NADP by ascorbate in aqueous media and that the reaction required the presence of partially purified Fd. Later work[15, 16] with purified preparations showed that Fd did not catalyze the photoreduction of pyridine nucleotides with either hematoporphyrin or chlorophyllin *a* as the photocatalyst when Fd was present either alone or together with the NADP reductase. With either photocatalyst, only the NADP reductase was required for NADP photoreduction in this system. The photoreduction of NAD or NADP in chloroplasts required both the presence of Fd and the NADP diaphorase as previously noted. No evidence was presented for the photoreduction of Fd in the porphyrin (or chlorophyll)-ascorbate system. Fd, however, has been noted as the earliest chemically isolatable reductant formed during conversion of electromagnetic energy into chemical energy in photosynthesis.[25] None of these previous studies have provided evidence that Fd might interact directly with chlorophyll in the intact chloroplast as the primary electron acceptor. The work to be described in this paper demonstrates that Fd can be photoreduced in the presence of a porphyrin and light with an electron donor.

Materials and Methods.—Spinach Fd was prepared by a procedure[17] which involved acetone extraction, according to San Pietro and Lang,[1] followed by chromatography on DEAE-cellulose based on the method of Lovenberg *et al.*,[18] for Clostridial Fd and finally by chromatography on Sephadex G-75. The protein was stored at −20°C. During the course of these experiments, only a small decrease

in the visible absorption spectrum of the protein was observed, following frequent freezing and thawing. Coproporphyrin I tetramethylester was obtained from Calbiochem. Approximately ten mg of the porphyrin ester were dissolved in 1 ml of 25 per cent HCl and stored in the dark for 48 hours. Following hydrolysis, the solution was evaporated to dryness under vacuum. The coproporphyrin dihydrochloride was dissolved in a small volume of water containing six equivalents of sodium hydroxide and diluted to about 50 ml with 0.001 *M* Tris buffer, pH 7.9. The solution was filtered through Whatman No. 42 paper, divided into small aliquots, and stored frozen at −20°C. Dithioerythritol (DTE) was purchased from Cyclo Chemical Corporation and ascorbic acid was obtained from Fisher Scientific Company.

Photoreduction of Fd: The typical reaction solution contained 0.15 μmole of Fd, 0.015 μmoles coproporphyrin, 50 μmoles Tris buffer pH 7.95 (or 7.35), and 15 μmoles of DTE (or ascorbic acid), in a total volume of 3.0 ml. When ascorbic acid was used as the electron donor, an equivalent amount of a sodium hydroxide solution was added to the reaction solution. Ascorbic acid (or DTE) was placed in the sidearm of the Thunberg vessel and mixed with the reaction solution after the system was deaerated.

The reaction vessel was connected with flexible butyl rubber tubing to a vacuum line provided with a gas inlet valve. The solution was frozen in a dry ice and isopropanol bath and the system was evacuated. The system was then equilibrated, after thawing, with prepurified argon (Liquid Carbonic Co.) which was further purified by passage through a quartz tube containing MnO to remove traces of oxygen.[19] This procedure was repeated two times, after which the system was finally brought to vacuum at room temperature.

The reaction vessel was then removed from the vacuum line and placed in a specially constructed cuvette holder[20] having three sides open. One side faced the front wall of a Cary 14R spectrophotometer while the other two sides were open to the measuring beam. The anaerobic solutions were illuminated with a beam of exciting light directed through a hole in the front side of the sample compartment. The light source was a Sylvania D16 150-watt, 21.5-volt lamp, which was attached to a variable-voltage DC power supply. The actinic light was passed through 2 cm of water and then through a lens to focus the light on the sample cuvette. The intensity of the light incident on the reaction vessel was measured with a YSI-Kettering model 65 radiometer. Following illumination for various periods with the shutter to the phototube compartment closed, the actinic light was shut off, the shutter was opened, and the spectrum recorded.

Experimental Results.—Photoreduction of porphyrin: Mauzerall[21, 22] has described the photoreduction of uro-, copro-, diglycol-, and hematoporphyrins in the presence of a variety of electron donors including EDTA, glutathione, sodium dithionite, titanous chloride, and ascorbic acid. Figure 1 shows the progressive photoreduction of coproporphyrin at one light intensity used under the conditions described for the photoreduction of spinach Fd. In the presence of ascorbic acid or dithioerythritol, the photoreduction of coproporphyrin appeared to proceed to the colorless hexahydroporphyrin. The initial formation of the absorption maximum at 436 mμ has been identified with the dihydroporphyrin, while the very small increase in absorbance centered at 500 mμ is associated with the production

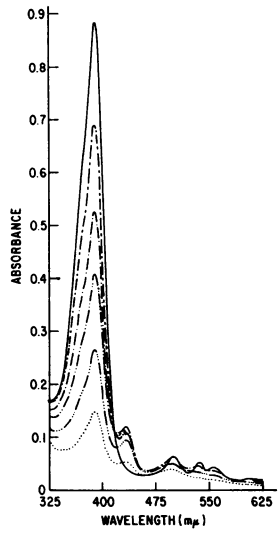

FIG. 1.—Progressive photoreduction of coproporphyrin in the presence of dithioerythritol and light. The reaction solution, prepared anaerobically, contained 0.015 μmole coproporphyrin, 50 μmoles Tris-HCl buffer pH 7.95, 15 μmoles DTE in a total volume of 3 ml. Spectra were recorded following brief periods of illumination at 7.3 × 10^6 ergs/cm²-sec.—— Initial spectrum; — - — - — 30 sec; —·—·—60 sec; —··— ··90 sec; —···—···—150 sec; .....240 sec.

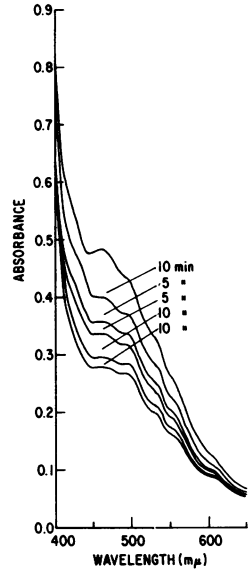

FIG. 2.—Progressive photobleaching of an anaerobic solution containing 0.150 μmole Fd, 0.015 μmole coproporphyrin, 50 μmoles Tris-HCl buffer pH 7.35, 15 μmoles sodium ascorbate in 3 ml. Spectral changes were recorded at progressively increasing intervals of illumination at 4.0 × 10^6 ergs/cm²-sec. Total illumination period = 40 min.

of the tetrahydroporphyrin. However, even after the first short illumination, the dihydro- and tetrahydroporphyrins represented only a small fraction of the total porphyrin reduced, based on their published extinction coefficients.[22]

Photobleaching of Fd: Figure 2 shows the progressive bleaching of a solution of Fd containing coproporphyrin and ascorbic acid following increasing periods of illumination. In Figure 3, the changes in per cent absorbancy of Fd at 420 and 460 mμ are plotted as a function of the time of illumination. Further illumination resulted in almost no change in absorbance at 420 mμ and only a small decrease in absorbance at 460 mμ. Fry *et al.*[23] have reported that the decrease in absorbance following reduction of Fd with sodium dithionite corresponds to a loss of about 49 and 55 per cent of the original absorbance at 420 and 460 mμ, respectively. Absorbance changes of 50 per cent at 420 mμ and 54 per cent at 463 mμ were reported by Whatley *et al.*,[4] also, for the reduction with dithionite. The maximum extent of photobleaching in the presence of coproporphyrin and ascorbic acid observed corresponds to 46 and 47 per cent bleaching at 420 and 460 mμ, respectively.

In the absence of light there is no change in the initial spectrum of an anaerobic solution containing Fd, coproporphyrin, and ascorbic acid. Illumination of a solution of Fd and ascorbate in the absence of porphyrin results in no decrease in

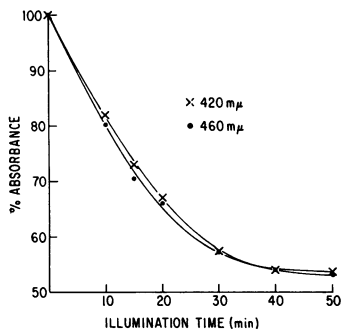

FIG. 3.—Spectral changes in per cent absorbance of Fd at 420 and 460 mμ as a function of time of anaerobic illumination. The per cent change in absorbance of Fd was calculated from the change in absorbance after subtracting the contribution of coproporphyrin to the total absorbance at 420 and 460 mμ.

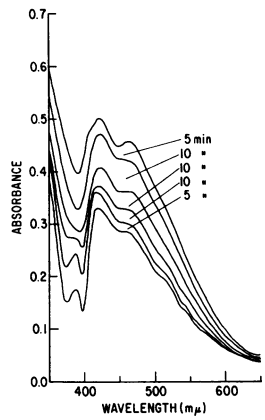

FIG. 4.—Photoreduction (anaerobic) of Fd. Spectral changes were recorded at indicated intervals (total, 40 min) as a difference spectrum between a sample cuvette containing 0.150 μmole Fd, 0.015 μmole coproporphyrin, 50 μmoles Tris-HCl buffer pH 7.35, 15 μmoles sodium ascorbate in 3 ml, and a reference cuvette containing coproporphyrin and Tris buffer at the same concentration. Illumination at 4.0×10^6 ergs/cm²-sec.

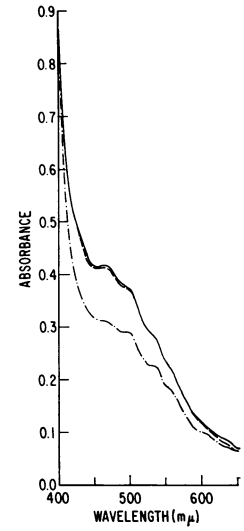

FIG. 5.—Reoxidation of Fd by oxygen in the dark following partial photoreduction. Shown are spectral changes in Fd on illumination of an anaerobic solution, containing 0.15 μmole Fd, 50 μmoles Tris-HCl buffer pH 7.95, 0.015 μmole coproporphyrin, 15 μmoles DTE, in 3 ml, followed by addition of oxygen in the dark. Spectra were recorded before (———) and after (—·—·—) illumination; then after addition of oxygen (1 atm pressure) the reaction vessel was rotated several times and the spectrum noted (———). Illumination at 4.0 $\times 10^6$ ergs/cm²-sec for 20 min.

the absorption spectrum of Fd. Likewise, illumination of an anaerobic solution of Fd plus coproporphyrin without ascorbic acid causes no bleaching of either the Fd or porphyrin spectrum. Figure 4 shows the decrease in absorbance of Fd following illumination of the complete anaerobic system. The reference solution contained coproporphyrin at a concentration equal to that of the sample in Tris buffer. The bleaching at about 390 mμ is in part due to a partial photoreduction of the coproporphyrin.

Reoxidation of bleached Fd with oxygen: Whatley *et al.*[4] and Fry *et al.*[23] have reported that Fd photoreduced by illuminated chloroplasts was stable and could be largely reoxidized by NADP even after a considerable dark period. Likewise, if Fd was reduced by dithionite, it could be reversibly oxidized to a large extent with oxygen.[23] Bleaching of Fd following illumination in the above system was also reversible upon exposure to oxygen. Figure 5 shows that after photobleaching of a solution of Fd to the extent of *ca.* 49 per cent reduction of the Fd, addition of oxygen caused complete reversal.

Discussion and Conclusions.—The reduction potential of DTE is reported[24]

to be −0.33 v at pH 7.0 and that of Fd at pH 7.55, −0.432 v. DTE is, however, present in 100-fold excess over that of Fd in the above reaction solution and would be expected to partially reduce Fd in the absence of porphyrin and light. However, DTE reacts only very slowly with Fd in times much longer than required for the photoreduction, though it is an effective donor for the light-induced reduction.

As noted above, the fact that Fd can function as an electron acceptor of illuminated chloroplasts and subsequently as a reductant for pyridine nucleotides has been established. Ferredoxin has tentatively been suggested as the primary electron acceptor (System I) in various schemes[4, 25, 26] of green plant photosynthesis. However, while previous reports have demonstrated that Fd can be reduced chemically by a variety of electron donors, including sodium dithionite, hydrogen and hydrogenase, and hydrogen and platinum asbestos, there have been no data presented to show that Fd could interact directly chlorophyll in the photoactive chloroplast. Coproporphyrin and chlorophyll, as cyclic tetrapyrroles, have common structural features and in addition have previously been shown to be active in photosensitizing the reduction of various dyes[27] and pyridine nucleotides (see above). The above results which demonstrate the photoreduction of Fd in the presence of a chlorophyll-like compound as the photocatalyst support the suggestion that Fd could act as a primary electron acceptor in photosystem I of green plants.

Summary.—In the presence of coproporphyrin as catalyst, Fd can be photoreduced by electron donors (ascorbic acid and DTE). This reduction is reversible by oxygen in the dark. The catalyst is essential for the photoreduction, but not for the reverse dark oxidation.

Note added in proof: We have recently received a personal communication from Mrs. Karolyn Eisenstein and Dr. Jui H. Wang describing the photoreduction of a bacterial ferredoxin in a quite similar system. Their results will be published shortly.

Abbreviations: PPNR, photosynthetic pyridine nucleotide reductase; Fd, spinach ferredoxin; NAD, oxidized diphosphopyridine nucleotide; NADP, oxidized triphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetate; DTE, dithioerythritol.

* National Institutes of Health postdoctoral fellow (grant 5-F2-GM-24,040-02).

† This research was supported by grants from the National Institutes of Health (HD-01262) and the National Science Foundation (GB 2892).

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