PHOTOREDUCTION OF FERREDOXIN-NADP IN THE PRESENCE AND ABSENCE OF FERREDOXIN-REDUCING SUBSTANCE (FRS)

Harry Y. Tsujimoto, Richard K. Chain, and Daniel I. Arnon
Department of Cell Physiology, University of California
Berkeley, California 94720

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Summary: Preparations of ferredoxin-reducing substance (FRS) were obtained from spinach chloroplasts within the elution volume range and with the spectral characteristics described by Yocum and San Pietro (8). However, no support was found for the view that FRS is the primary electron acceptor of Photosystem I. The FRS-depleted chloroplast fragments retained their Photosystem I activity, which was not enhanced by the addition of FRS. No evidence was found for a prior photoreduction of FRS by chloroplasts followed by a dark reduction of ferredoxin and NADP by reduced FRS. The FRS-depleted chloroplast fragments were found to retain and to photoreduce bound ferredoxin upon illumination by Photosystem I light at 25°K. These results suggest that the role of a primary electron acceptor of Photosystem I ascribed to FRS may belong to bound ferredoxin.

When chloroplast ferredoxin was found to have a redox potential E_m = -432 mV at pH 7.55 (1), this iron-sulfur protein became identified as the most electronegative component isolated from the photosynthetic apparatus, with a reducing power about equal to that of molecular hydrogen (1-3). The possible existence of more negative electron carriers was later suggested (4-6) from experiments on the photoreduction by chloroplasts of strongly electronegative nonphysiological dyes but such suggestions remained speculative until the postulated carrier(s) could be isolated and/or chemically characterized.

Yocum and San Pietro (7,8) reported the isolation from spinach chloroplasts of a "ferredoxin-reducing substance (FRS)" that functions as the primary electron acceptor at the reducing terminus of Photosystem I. Photoreduced FRS served as the reductant for the subsequent dark reduction of ferredoxin and NADP. Chloroplast fragments from which FRS was removed were incapable of photoreducing ferredoxin and NADP or such nonphysiological substitutes as methyl viologen.

More recently, P700, the reactive chlorophyll *a* component of Photosystem I,

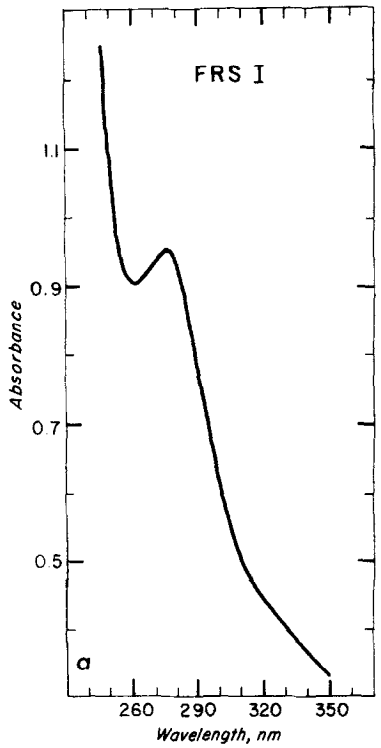
was identified as the reaction partner for the photoreduction of FRS (9). This identification was in conflict with recent findings from this laboratory that P700 is the reaction partner in the photoreduction of a bound iron-sulfur protein of the ferredoxin type which has been identified as the primary electron acceptor in Photosystem I (10-12). FRS could not be identical with bound ferredoxin since (i) contrary to the equivalent amounts of non-heme iron and labile sulfide associated with bound ferredoxin (10), the prosthetic group of FRS contained neither iron nor sulfur and appeared to be a flavonoid (8); (ii) FRS but not the bound ferredoxin was removable from chloroplasts by sonication (8); (iii) the absorption spectrum of FRS was devoid of features characteristic of ferredoxins; and (iv) the low molecular weight (about 4600) of FRS (9) contrasted with molecular weights, around 12,000, characteristic of plant ferredoxins.

In the present investigation, FRS preparations were isolated from spinach chloroplasts by the method of Yocum and San Pietro (8). However, the "FRS-depleted" chloroplast fragments retained their Photosystem I activity and their content of bound ferredoxin was the same as that of the control chloroplasts. No evidence was found for a prior photoreduction of FRS by chloroplasts followed by a dark reduction of ferredoxin and NADP by reduced FRS.

These results are inconsistent with the view that FRS is the primary electron acceptor for Photosystem I but are consistent with the view that that function may belong to bound ferredoxin.

Methods

FRS was isolated and the FRS-depleted chloroplast fragments were prepared by the improved method of Yocum and San Pietro (8). The activity of the FRS-depleted chloroplast fragments was tested as described earlier by Yocum and San Pietro (7). Plastocyanin was prepared from spinach leaves by the method of Katoh et al. (13). Procedures previously reported by this laboratory were used for the preparation of ferredoxin (14) and ferredoxin-NADP reductase (15), for the estimation of chlorophyll (16), and for the detection of photoreduced bound



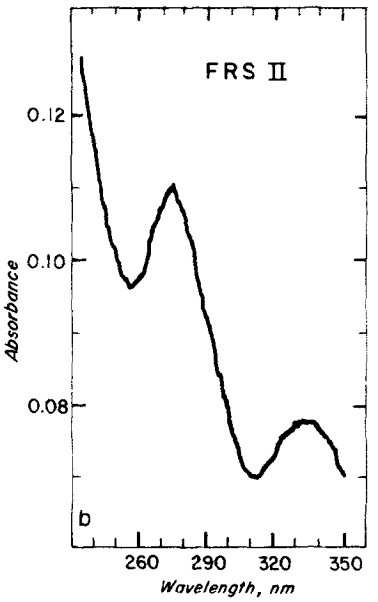


Fig. 1a. Absorption spectrum of FRSI. Experimental conditions: gas phase, air; temperature, 20°; light path, 5 mm.
Fig. 1b. Absorption spectrum of FRSII. Experimental conditions as in Fig. 1a except that a 2-mm light path was used.

ferredoxin by electron paramagnetic resonance (EPR) spectroscopy (10-12).

Results and Discussion

The absorption spectrum of FRS shown by Yocum and San Pietro (8) had a single peak at 270 nm but they also observed in some preparations a secondary absorption peak at 330 nm. By collecting the eluate from the Sephadex G-75 column in small fractions of about 5-10 ml, we separated [within the reported elution range for FRS (8)] two preparations. The early fractions yielded, when concentrated by lyophilization, a preparation that had an absorption spectrum with a single peak around 270 nm (Fig. 1a). Later fractions yielded a preparation with a double-peak absorption spectrum, one peak around 270 nm and another around 330 nm (Fig. 1b). Since the spectra of both preparations corresponded to those attributed to FRS (8), the two preparations were

Table 1

EFFECT OF FRS PREPARATIONS ON NADP PHOTOREDUCTION BY CHLOROPLAST FRAGMENTS

Treatment	$NADPH_2$ Formed
	μmoles/mg chl/hr
Control	480
+0.2 ml FRSI	460
+0.4 ml FRSI	480
+0.2 ml FRSII	500
+0.4 ml FRSII	480
+0.1 ml FRSI + 0.1 ml FRSII	480
+0.2 ml FRSI + 0.2 ml FRSII	500

The control contained (per 1.0 ml) chloroplast fragments (equivalent to 10 μg chlorophyll), ferredoxin-NADP reductase (A_{456} = 0.0080 cm^{-1}) and the following in μmoles: Tricine buffer, pH 6.0, 200; 3-(3',4'-dichlorophenyl)-1,1-dimethyl urea (DCMU), 0.010; ascorbate, 10; 2,6-dichlorophenol indophenol (DPIP) 0.10; plastocyanin, 0.010; spinach ferredoxin, 0.010; and NADP, 2.0 Where indicated, FRSI (A_{275} = 0.65 cm^{-1}) and FRSII (A_{275} = 0.55 cm^{-1}) were added. Gas phase, air; temperature, 20°; illumination, wide-band red light (2.5 x 10^5 $ergs/cm^2$ per sec). The chloroplast fragments were depleted in FRS (see Methods).

designated, respectively, FRSI and FRSII and were tested for their effect on the photoreduction of ferredoxin and NADP by FRS-depleted chloroplasts.

The addition of FRSI and FRSII, either singly or jointly, to chloroplast fragments from which FRS was removed did not stimulate their rate of NADP photoreduction (Table 1). The photoreduction of NADP was independent of added FRS but was otherwise similar to that reported by Yocum and San Pietro (7) in being totally dependent on added ferredoxin, ferredoxin-NADP reductase, plastocyanin, and ascorbate. The addition of DPIP stimulated but was not absolutely essential for the reduction of NADP.

Since the ineffectiveness of added FRS might have conceivably resulted from its incomplete removal from the chloroplasts, special attention was given to the evidence from a two-stage experiment (7), similar in design to that used earlier (17) to demonstrate that photoreduction of ferredoxin precedes the reduction of NADP by chloroplasts. In the first stage, substrate amounts of FRS were photoreduced in the absence of ferredoxin; in the second stage, the reduced FRS was used to reduce in the dark either substrate amounts of ferredoxin or substrate amounts of NADP in the presence of catalytic amounts of ferredoxin (7).

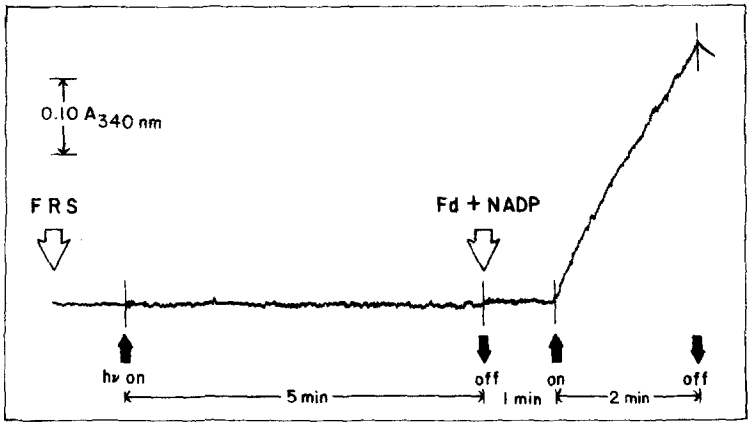


Fig. 2. Preillumination of FRS and subsequent photoreduction of ferredoxin and NADP. After preilluminating FRS for 5 min, the light was turned off and the electron acceptor system (Fd + NADP) was tipped in from the sidearm. The reaction mixture, placed in the main compartment of the Thunberg cuvettes (10-mm light path), contained, in a volume of 2 ml, chloroplast fragments (equivalent to 20 µg chlorophyll), FRSII, $A_{275} = 1.0\ cm^{-1}$, and the following in µmoles: Tris-HCl buffer, pH 7.5, 150; DCMU, 0.02; sodium ascorbate, 10; DPIP, 0.1; and plastocyanin, 0.02. The electron acceptor system placed in the sidearm contained in a volume of 0.2 ml: ferredoxin, 0.02 µmoles; ferredoxin-NADP reductase, $A_{456} = 0.016\ cm^{-1}$; and NADP, 2 µmoles. Gas phase, argon; temperature, 20°; illumination, 650-nm monochromatic light (2×10^5 ergs/cm^2/sec). NADP reduction was measured by the increase in absorbance at 340 nm. A reference cuvette was treated in the same manner except for illumination.

Our experiments to reproduce a similar two-stage reduction of FRS and ferredoxin-NADP are illustrated in Fig. 2. Substrate amounts of FRS (prepared from about 2 kg of leaves for each experiment) were illuminated anaerobically in Thunberg cuvettes for 5 min in the absence of the electron acceptor system, consisting of ferredoxin, ferredoxin-NADP reductase, and NADP. At the end of the 5-min illumination period, the electron acceptor system was tipped from the sidearm into the main compartment but no reduction of NADP occurred in the dark. Similar results were obtained by preilluminating either FRSI or FRSII. Thus, no evidence was obtained for an accumulation of reducing power by a preillumination of chloroplast fragments and FRS in the absence of ferredoxin.

That the chloroplast fragments were photochemically viable was demonstrated by an active reduction of NADP as a result of a second illumination after the electron acceptor system was added to the reaction mixture (Fig. 2).

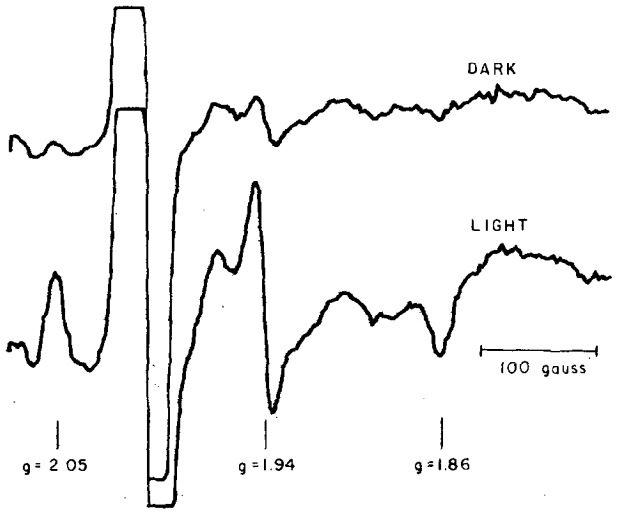


Fig. 3. Light-induced EPR changes of bound ferredoxin in FRS-depleted chloroplasts at 25°K. Chlorophyll concentration was 2 mg/ml. Approximately 5 μmoles of sodium ascorbate were added to the chloroplast suspension prior to freezing. The sample was illuminated at 25°K with 715-nm monochromatic light (2×10^4 ergs/cm^2/sec) for 30 sec. First-derivative EPR spectra were recorded at 25°K as previously described (10-12).

To recapitulate, by using the improved method for isolation of FRS (8) we obtained FRS preparations that were eluted from the Sephadex G-75 gel column in the described volume range and had the described spectral characteristics. However, these FRS preparations did not enhance the rate of NADP reduction by chloroplasts from which they were prepared. Moreover, there was no evidence that preilluminated FRS can serve as a reductant for ferredoxin in the dark.

As already stated, recent work (10-12), confirmed in several laboratories (18-20), has indicated that the primary electron acceptor of Photosystem I is an iron-sulfur protein of the ferredoxin type that remains bound in the membrane system of chloroplasts after the soluble ferredoxin is removed. It would be expected, therefore, that the FRS-depleted chloroplast fragments which retained a Photosystem I activity also retained their bound ferredoxin. Evidence on this point is presented in Fig. 3. Illumination of the chloroplast fragments by Photosystem I light (715 nm) at 25°K gave an EPR spectrum with signals at $g = 2.05$, $g = 1.94$, and $g = 1.86$ that are characteristic of photoreduced bound ferredoxin (10-12). The magnitude of the signals was not affected by removal of FRS.

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