FERREDOXIN REDUCING SUBSTANCE (FRS) FROM SPINACH*

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Received June 30, 1969

Summary: The requirement for a heretofore unidentified chloroplast component for NADP photoreduction by chloroplast fragments is established. This component, termed "ferredoxin reducing substance (FRS)," is required also for the photoreduction of methyl viologen and, in the reduced form, serves as the reductant for the dark reduction of NADP or ferredoxin. These data suggest that FRS is the primary acceptor for photosystem 1 of photosynthesis.

The functionality of a primary acceptor, other than ferredoxin, at the reducing terminus of photosystem 1 of the photosynthetic electron transport chain has been shown indirectly by a number of investigators. Without identification of the primary acceptor, it has nonetheless been possible to demonstrate its presence since chloroplast fragments devoid of ferredoxin retain the ability to photooxidize reduced cytochrome f (Chance et al, 1965) and, in addition, whole chloroplasts can photoreduce a series of viologen dyes with E_o' values as low as - 0.7 volt (Zweig and Avron, 1965; Kok et al, 1965; Black, 1966). In the latter investigations, the characteristic potential of the primary acceptor was estimated to be about - 0.5 to - 0.55 volt based on the concentrations of oxidized and reduced viologen present at equilibrium.

More recently, Fujita and Myers (1967) and later Fujita and Murano (1967) isolated a material called "cytochrome reducing substance (CRS)" and demonstrated that cytochrome c reduction, DCMU sensitive photophosphorylation (pseudocyclic), and photooxidation of reduced DPIP and plastocyanin were influenced by this material. Based on these observations, they concluded that CRS might exert its influence on electron flow between P700 and ferredoxin or oxygen.

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* This research was supported in part by a Research Grant (GM 16314 to A.S.P.) from the National Institutes of Health, United States Public Health Service.

Last year, Yocum et al (1968) reported the separation of disrupted grana by sucrose density gradient centrifugation into fractions enriched either in photosystem 1 or 2 activity. During these experiments, they noted the presence of a yellow material, called "lipoidal factor", which had no effect on photosystem 2 activity but markedly stimulated NADP photoreduction by photosystem 1. However, the site of involvement of this factor was not investigated further.

We will present evidence that this factor exerts its influence at the level of the primary acceptor for photosystem 1. Since it is required absolutely for the photoreduction of NADP, ferredoxin and methyl viologen by chloroplast fragments, we have termed it "ferredoxin reducing substance (FRS)". Further, photoreduced FRS can serve as the reductant for the subsequent dark reduction of NADP or ferredoxin.

Materials and Methods: Chloroplast fragments and FRS were prepared by sonic disruption of washed chloroplasts and sucrose density gradient centrifugation as described previously (Yocum et al, 1968). Ferredoxin was purified by DEAE chromatography (Losada and Arnon, 1964); ferredoxin-NADP reductase by the method of Shin et al (1963); and Euglena cytochrome-552 and spinach plastocyanin as described by Perini et al (1964) and by Katoh (1962), respectively. Chlorophyll concentration was determined by the method of Arnon (1949). All spectrophotometric measurements were made with a Hitachi-Coleman recording spectrophotometer, Model 124.

Results and Discussion: The requirements for NADP photoreduction by the chloroplast fragments are indicated in Table I. It is clear that with these chloroplast fragments there is an absolute dependence on all the components; namely, ascorbate, DPIP, cytochrome-552 or plastocyanin, ferredoxin, ferredoxin-NADP reductase and FRS. It should be noted that with the exception of FRS, these component requirements are identical to those observed previously for NADP photoreduction by chloroplast fragments (enriched in photosystem 1) prepared by a variety of methods (see Vernon et al, 1966; Boardman, 1968). The apparent advantage of the chloroplast fragments used in our experiments is that they

Table I - Requirements for NADP Photoreduction by Chloroplast Fragments.

Reaction Mixture	Rate of NADPH Formation
Complete*	82
- Ascorbate	0
- DPIP	1
- Cytochrome-552 or Plastocyanin	0
- Ferredoxin	0
- Ferredoxin-NADP Reductase	5
- FRS	6

*The complete system contained 20 μmoles of ascorbate, 0.2 μmole of DPIP, 0.5 μmole of NADP, 17 micrograms of chlorophyll as chloroplast fragments, 200 μmoles of Tricine buffer, pH 6, 0.2 ml of FRS, and saturating amounts of cytochrome-552 or plastocyanin, ferredoxin and ferredoxin-NADP reductase in a final volume of 1.0 ml. Photosystem 2 was inhibited by the addition of DCMU at a final concentration of 10 μM. The reaction mixture was illuminated for five minutes and NADPH formation determined by the increase in absorbance at 340 nm. The data are presented as μmoles of NADPH formed per hour per milligram of chlorophyll.

exhibit a complete dependence on the heretofore undiscovered factor, FRS. None of the previously described chloroplast fragments exhibit this dependence.

The relationship between the rate of NADP photoreduction and FRS concentration is shown in Figure 1. In these experiments all the components of the reaction mixture were kept constant except FRS concentration which was varied. At low concentrations, there is a linear relationship between NADP photoreduction and FRS concentration; at higher concentrations of FRS, the rate of NADP photoreduction is independent of FRS concentration. This type of relationship indicates that FRS functions catalytically; that is, the curve in Figure 1 is characteristic of saturation kinetics typical of catalytic function.

While the results described thus far establish the requirement for and

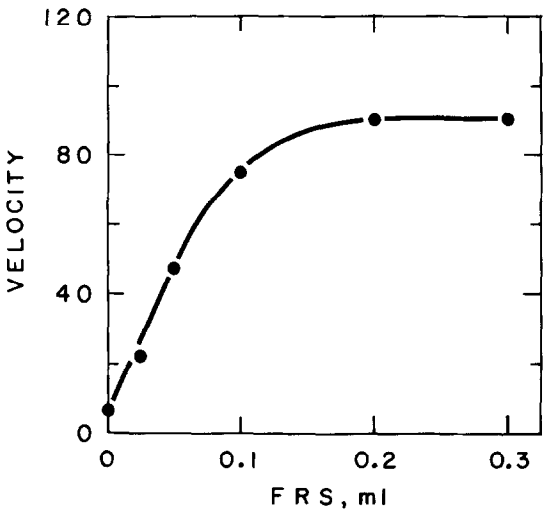


Figure 1- Effect of FRS on NADP Photoreduction by Chloroplast Fragments. Conditions were as described in Table I. Velocity is given as μmoles of NADPH formed per hour per milligram of chlorophyll.

catalytic action of FRS, they do not indicate anything about the site of function of FRS. Two possibilities exist for the site of function of FRS; namely, the oxidizing or reducing side of photosystem 1. It was possible to decide between these two alternatives by carrying out the reduction of NADP and/or ferredoxin in two stages; the first was light-dependent, whereas the second was not. In these experiments (Table II), chloroplast fragments were illuminated under anaerobic conditions in the presence of ascorbate, DPIP, plastocyanin and a substrate amount of FRS. After cessation of illumination, either NADP and a catalytic amount of ferredoxin and ferredoxin-NADP reductase (Table II, Expt. 1) or a substrate level of only ferredoxin (Table II, Expt. 2) was added and the formation of NADPH or reduced ferredoxin measured spectrophotometrically. The formation of NADPH as the product of the dark reaction was confirmed enzymatically with glutamic dehydrogenase by measuring the decrease in the absorbance at 340 nm accompanying the enzymatic oxidation of NADPH. Reduced ferredoxin formed in the dark (Table II, Expt. 2) was oxidized by aeration and the resultant change in absorbance at 430 nm is the value reported in the table.

Table II - Dark Reduction of NADP and Ferredoxin by Photoreduced FRS.

Expt.	Substrate	$+\Delta A_{340}$	$-\Delta A_{430}$
1	NADP	0.10	-
2	Ferredoxin	-	0.07

The reaction mixture to be illuminated was placed in the main compartment of a Thunberg cuvette and contained 30-40 micrograms of chlorophyll as chloroplast fragments, 10 μmoles of ascorbate, 0.1 μmole of DPIP, 0.03 μmole of DCMU, 200 μmoles of Tris buffer, pH 7.5, 1.0 ml of FRS, plastocyanin and water to a final volume of 3 ml. In the sidearm was placed either NADP, ferredoxin and ferredoxin-NADP reductase (Expt. 1) or only ferredoxin (Expt. 2). After evacuation, the reaction mixture in the main compartment was illuminated for 5 minutes with a saturating intensity of white light. Following illumination, the contents in the sidearm were added and the amount of reduced NADP or reduced ferredoxin formed in the dark was determined as described in the text.

Table III - Dependence on FRS of Methyl Viologen Photoreduction.

FRS	ΔA_{396}
-	0.00
+(0.5 ml)	0.11

The reaction mixtures were identical to those described in Table II (main compartment) with the exception that 0.6 μmole of methyl viologen was present and FRS was added only as indicated. Further, anaerobic conditions were maintained by inclusion of glucose, glucose oxidase, catalase and ethanol. Cysteine was used in place of ascorbate as the electron donor as described by Kok et al (1965). Illumination time was 20 minutes and reduced methyl viologen was detected as noted in the text.

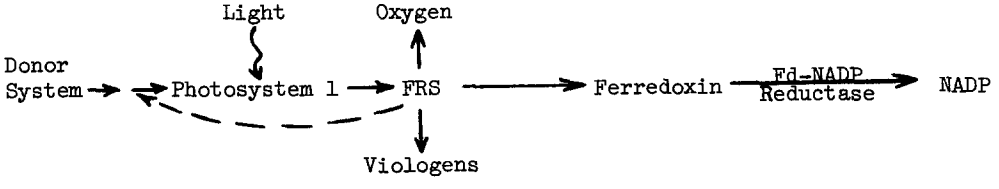
Inasmuch as previous investigators (Zweig and Avron, 1965; Kok et al, 1965; Black, 1966) had measured viologen reduction to indicate the functionality of the primary acceptor for photosystem 1, it was of interest to test the effect

of FRS on this activity. As shown in Table III, methyl viologen photoreduction occurs only in the presence of FRS. Because of the experimental difficulties in observing viologen reduction (due to autooxidizability of the reduced viologen), these experiments were performed at only a single FRS concentration. The formation of reduced methyl viologen was measured spectrally by scanning from 420 nm to 370 nm wherein the characteristic reduced viologen spectrum with a peak at 396 nm and a shoulder at 385 nm was resolved (Kok et al, 1965).

Since FRS also catalyzes the photooxidation of reduced DPIP, it was important to compare the catalytic properties of FRS and CRS. The CRS was prepared as described by Fujita and Meyers (1967) and it appears to possess some catalytic properties similar to those shown by FRS; e.g., catalysis of viologen photoreduction (Table IV) but not NADP photoreduction (Table I). It should be noted that the preparation of CRS involves heating at 100° for 6 minutes, whereas the preparation of FRS is carried out completely in the cold. Thus it may be premature to consider the question of possible identity between FRS and CRS without further documentation. This question is currently under investigation.

During the course of these experiments, Dr. A. Trebst kindly brought to our attention his earlier published work describing an antibody against an unknown factor required for NADP photoreduction by broken chloroplasts (Berzborn et al, 1966). He has tested our preparation of FRS and it did reverse the inhibitory effect of their antibody preparation (Trebst, personal communication). Moreover, their antibody preparation was inhibitory in our system and FRS reversed the inhibitory effect (Yocum and San Pietro, unpublished observations).

To explain these data, we consider the following scheme as a working hypothesis:



In the experiments reported herein, the FRS was prepared as described by Yocum et al (1968). In the interim, we have developed a better isolation procedure which appears at present to provide in good yield a preparation of FRS which is homogeneous electrophoretically. The availability of a purified preparation of FRS will allow us to determine its chemical and physical properties and to gain additional evidence to support our view that it is the primary acceptor for photosystem 1. Based on thermodynamic considerations, it is certainly reasonable that a component more reducing than ferredoxin functions in the photosynthetic electron transport pathway. The results of these investigations will be published elsewhere.

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