FERREDOXIN-ACTIVATED FRUCTOSE DIPHOSPHATASE IN ISOLATED CHLOROPLASTS

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Received August 28, 1967

The photoreduction of ferredoxin is a key event in photosynthesis. In isolated chloroplasts it was found to be coupled to photosynthetic phosphorylation (Tagawa et al., 1963) and to oxygen evolution (Arnon et al., 1964). Moreover, reduced ferredoxin transfers electrons to NADP (independently of light) by way of ferredoxin-NADP reductase (Shin and Arnon, 1965). $NADPH_2$ in turn acts as the hydrogen donor for the reduction of 1,3-diphosphoglyceraldehyde 3-phosphate (Warburg and Christian, 1939) -- a reaction that constitutes the only reductive step in carbon assimilation during photosynthesis (Calvin and Bassham, 1962).

The possibility that the key role of ferredoxin in photosynthesis may have an even more extensive basis was strengthened by recent evidence that ferredoxin acts directly as an electron donor in photosynthetic CO_2 assimilation in bacterial photosynthesis. In the photosynthetic bacteria, Chlorobium thiosulfatophilum (Evans and Buchanan, 1965; Buchanan and Evans, 1965) and Rhodospirillum rubrum (Buchanan et al., 1967), reduced ferredoxin supplies directly electrons for the synthesis of pyruvate from acetyl CoA and CO_2 and of α-ketoglutarate from succinyl CoA and CO_2. These new reactions led to evidence for the reductive carboxylic acid cycle as a new pathway for CO_2 assimilation in photosynthetic bacteria (Evans et al., 1966; Buchanan et al., 1967).

As part of an investigation of a possible direct role of ferredoxin in CO_2 assimilation by isolated chloroplasts, we have now obtained evidence for a requirement of reduced ferredoxin in the cleavage of fructose

1,6-diphosphate (FDP) in accordance with Equation 1.

(1) Fructose 1,6-diphosphate $\xrightarrow[\text{Ferredoxin}]{\text{Reduced}}$ Fructose 6-phosphate + P_i

The ferredoxin-activated fructose diphosphatase (FDPase) reaction required reduced ferredoxin, although no apparent oxidation or reduction is involved. The mechanism by which reduced ferredoxin activates FDPase is not yet clear, but, as discussed later, such activation may have an important regulatory action on photosynthetic carbon assimilation. No cofactors of the reaction, other than reduced ferredoxin, have been identified. Nor has any relation yet been found between the ferredoxin-activated FDPase to alkaline FDPase (Enzyme Commission No. 3.1.3.11) of chloroplasts (Racker and Schroeder, 1958; Losada et al., 1960). Alkaline FDPase has an absolute requirement for a divalent cation, such as magnesium, for activity but is not known to involve ferredoxin. The relationship of the new ferredoxin-activated FDPase to the magnesium-dependent alkaline phosphatase must await a purification of the new enzyme.

Results and Discussion

Table 1 shows the requirements for P_i (orthophosphate) release from FDP when either water (Exp. A) or an artificial donor system, ascorbate-dichlorophenol indophenol (Exp. B), was used as the electron donor for the photochemical generation of reduced ferredoxin. The spinach enzyme required spinach ferredoxin. Ferredoxin from the bacterium, Clostridium pasteurianum, or the non-physiological electron carrier, methyl viologen, was ineffective.

Table 1 also shows that the release of P_i from FDP was strictly dependent on FDP, chloroplast extract (containing the soluble enzymes of isolated chloroplasts) and on chloroplast particles (containing the photochemical apparatus). Reduced glutathione stimulated P_i release about 30%. Ferredoxin-activated FDPase appears to be specific for FDP. The related sugar phosphates, fructose 6-phosphate, fructose 1-phosphate, glucose diphosphate, glucose 6-phosphate, and glucose 1-phosphate, were not cleaved.

Table 1

REQUIREMENTS FOR FERREDOXIN-ACTIVATED FRUCTOSE DIPHOSPHATASE: FERREDOXIN REDUCED BY ILLUMINATED CHLOROPLASTS

Treatment	Pi released, μMoles	
	Exp. A	Exp. B
Complete	1.1	1.0
Glutathione omitted	0.8	0.8
Ferredoxin omitted	0.0	0.1
Fructose-di-phosphate omitted	0.0	0.1
Chloroplast extract omitted	0.0	0.0
Chloroplast particles omitted	0.0	0.1
Complete, ferredoxin not reduced	0.0	0.1

In Exp. A, the complete system contained chloroplast extract (equivalent to 1 mg chlorophyll, see below), chloroplast particles (0.2 mg chlorophyll), spinach ferredoxin (0.2 mg) and the following in μmoles: Tris (tris[hydroxymethyl]aminomethane) buffer, pH 8.0, 200; neutralized reduced glutathione, 5; sodium fructose 1,6-diphosphate, 6. In Exp. B, the complete system contained, in addition to the components listed for Exp. A, sodium ascorbate, 20 μmoles, and 2,6-dichlorophenol indophenol, 0.2 μmoles. In Exp. B, chloroplast particles were heated 5 min at 55° to destroy their capacity for oxygen evolution. Final volume, 3.0 ml. Gas phase was argon. Light intensity (yellow light) was 10,000 lux. The reaction was carried out at 20° in Warburg vessels containing FDP in the side-arm. After 10 min equilibration with argon, vessels were preilluminated 10 min; FDP was added from the side-arm, and the reaction was continued for 20 min. In the complete treatment, ferredoxin not reduced, the reaction was carried out without illumination. The reaction was stopped by adding 0.5 ml 10% trichloroacetic acid, and after centrifuging off the precipitate, aliquots were analyzed for P_i by a modified Fiske-Subbarow procedure (Sumner and Somers, 1949).

Chloroplast extract (Whatley et al., 1956) was made by suspending chloroplasts prepared in isotonic sorbitol media in a 1 to 10 dilution of the preparative solution (Kalberer et al., 1967).

The products, other than orthophosphate, of the FDPase reaction were identified with uniformly labeled C^{14}-FDP as fructose 6-phosphate and glucose 6-phosphate by paper chromatography (Kalberer et al., 1967) and radioautography of the phosphorylated sugars and of the enzymically formed dephosphorylated free sugars and 6-phosphogluconic acid derivatives.

Fig. 1 shows the effect of ferredoxin concentration on P_i release from FDP when ferredoxin was reduced photochemically with water as the electron donor. P_i release was proportional, up to 50 μg, to the amount of added ferredoxin. Fig. 2 shows the time course of P_i release with and without

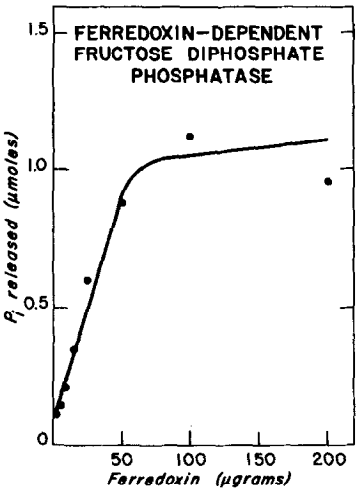

Fig. 1. Effect of ferredoxin concentration on orthophosphate (P_i) release from fructose diphosphate. Except for the variable ferredoxin concentration, experimental conditions were as given for Table 1.

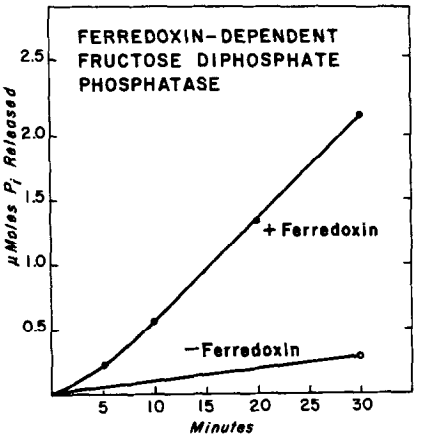

Fig. 2. Time course of ferredoxin-activated P_i release from fructose diphosphate. Experimental conditions were as given for Table 1.

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added ferredoxin. After a lag period of 5 min, P_i release proceeded linearly for at least 30 min in the presence of ferredoxin. After 30 min, added ferredoxin gave an almost ten-fold increase in the P_i released. In the experiments discussed so far, the reaction vessels were preilluminated for 10 min prior to addition of FDP. The preillumination shortened, but did not completely eliminate, the lag period seen in Fig. 2.

Tagawa and Arnon (1962) showed that H_2 (in the presence of a bacterial hydrogenase) can reduce ferredoxin in the dark, and thereby, replace illuminated chloroplasts as a source of reducing power. Table 2 shows that H_2 and a partially purified hydrogenase from C. pasteurianum generated the reduced ferredoxin needed for P_i release from FDP. This experiment showed that the

Table 2

REQUIREMENTS FOR FERREDOXIN-ACTIVATED FRUCTOSE DIPHOSPHATASE: FERREDOXIN REDUCED BY H_2 AND A HYDROGENASE FROM CLOSTRIDIUM PASTEURIANUM

Treatment	P_i Released μMoles
Complete	2.9
Ferredoxin omitted	0.4
Fructose-di-phosphate omitted	0.2
Chloroplast extract omitted	0.0
Hydrogenase omitted	0.0
Complete, ferredoxin not reduced	0.1

Experimental conditions were as given for Table 1 (Exp. A) except that chloroplast particles were omitted, hydrogenase (3 mg protein) from C. pasteurianum was added, and H_2 replaced argon as the gas phase (with the exception of the complete treatment, ferredoxin not reduced, which was under argon). Hydrogenase was obtained from C. pasteurianum cell-free extracts, which were prepared and treated to remove ferredoxin essentially as described by Bachofen et al. (1964), by heating 10 min at 60° under H_2 and centrifuging off the precipitate.

ferredoxin-activated FDPase is a soluble enzyme present in chloroplast extract and is independent of the chlorophyll-containing grana. Table 2 shows that the requirements for the phosphatase reaction in the H_2-hydrogenase system were the same as in the photochemical system (Table 1).

In 1954, Krebs elucidated the importance of FDPase in regulating gluconeogenesis. Until now, the alkaline magnesium-dependent FDPase was the only phosphatase known to function specifically with FDP in chloroplasts. The new evidence points to the existence in chloroplasts of another FDPase which is activated by reduced ferredoxin. Since ferredoxin is reduced by

photochemical reactions *in vivo*, the new phosphatase might serve as a light-actuated regulator of carbohydrate metabolism in green plants.

Acknowledgments

This research was aided by grants from the National Institute of General Medical Sciences, Office of Naval Research and the National Science Foundation.

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