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A NEW PROTEIN FACTOR FUNCTIONAL IN THE FERREDOXIN-INDEPENDENT LIGHT ACTIVATION OF CHLOROPLAST FRUCTOSE 1,6-BISPHOSPHATASE

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Summary: A protein purified from chloroplasts (the "new protein factor") activated Fru-P_2ase in a photochemical reaction that depended only on chloroplast membranes. The results suggest that chloroplasts utilize the newly found mechanism for the photoregulation of Fru-P_2ase in addition to the recently described ferredoxin/thioredoxin system.

In 1967, we reported that fructose 1,6-bisphosphatase (Fru-P_2ase) of spinach chloroplasts is activated by light via soluble ferredoxin (1). Later experiments revealed that ferredoxin did not activate Fru-P_2ase directly but that an additional protein fraction (designated the "protein factor") was required (2). On purification, the protein factor was separated into two components (3) that were identified as chloroplast thioredoxin and ferredoxin-thioredoxin reductase (4). Recently, the chloroplast thioredoxin fraction was itself separated into two different thioredoxin components--thioredoxins f and m (5-7).

There is now evidence from several laboratories that thioredoxin, ferredoxin, and ferredoxin-thioredoxin reductase constitute a general system of light-actuated enzyme regulation (7). In this system, photoreduced ferredoxin is used to reduce, via ferredoxin-thioredoxin reductase, the two chloroplast thioredoxins. The reduced thioredoxins, in turn, selectively activate key regulatory enzymes: thioredoxin f preferentially activates Fru-P_2ase, other regulatory members of the reductive pentose phosphate cycle (phosphoribulokinase, NADP-glyceraldehyde 3-phosphate dehydrogenase, sedoheptulose 1,7-bisphosphatase), and certain biosynthetic enzymes (phenylalanine ammonia lyase and PAPS sulfotransferase); thioredoxin m preferentially activates NADP-malate dehydrogenase (5-8). Thioredoxins may thus be visualized to act as regulatory messengers whereby light is used for

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the regulation of enzyme activity during photosynthesis.

We now report that thioredoxins are not the only chloroplast proteins that can serve in a regulatory capacity. A protein fraction purified from chloroplasts has been found to activate Fru-P_2ase photochemically in the absence of the components of the ferredoxin/thioredoxin system. The results suggest that this protein fraction (designated the "new protein factor") functions as an alternate (ferredoxin-independent) mechanism whereby light can modulate Fru-P_2ase activity.

METHODS

Assay for the New Protein Factor. The new protein factor was assayed by measuring its capacity to promote the light-dependent activation of chloroplast Fru-P_2ase in a reaction mixture which, except for the omission of ferredoxin, thioredoxin and ferredoxin-thioredoxin reductase, was similar to the one used for measuring ferredoxin-linked Fru-P_2ase activation. The reaction was carried out at 20° in Warburg vessels containing, in the side arm, 6 µmol of fructose 1,6-bisphosphate and, in the main compartment (in a final volume of 1.5 ml), 28 µg of Fru-P_2ase, thrice-washed chloroplast membrane fragments (1) [or Nostoc muscorum membrane fragments, C-144, prepared according to Arnon et al. (9)] equivalent to 0.1 mg of chlorophyll, new protein factor as needed, and the following (in µmol): Tris-HCl buffer (pH 8.0), 100; $MgSO_4$, 1; sodium ascorbate, 10; and dichlorophenol indophenol, 0.1. Vessels were equilibrated with nitrogen for 5 min and preincubated for the same time in saturating light (20,000 lux). The reaction was started by the addition of fructose 1,6-bisphosphate from the side arm and continued for 30 min under illumination. The reaction was stopped by adding 0.5 ml of 10% trichloroacetic acid. After the precipitate was removed by centrifugation, inorganic phosphate was estimated by a modified Fiske-Subbarow procedure (1).

Other Assays. Fru-P_2ase was assayed in the presence of 10 mM $MgSO_4$ by the colorimetric procedure described earlier (10). Ferredoxin activity was measured by the ability to promote the reduction of NADP by illuminated chloroplast membranes (11). Thioredoxin *f*, thioredoxin *m*, and ferredoxin-thioredoxin reductase were assayed as described previously (6). The ferredoxin-thioredoxin reductase activities shown below have been corrected for the activity of the new protein factor.

Analytical Methods. A procedure described earlier was used for the estimation of chlorophyll (11). Protein was estimated, in the presence of 2-mercaptoethanol, by a modified Lowry procedure (12).

Purification of Known Proteins. Previously described procedures were used for the purification of ferredoxin (11), thioredoxin *f* (5), and Fru-P_2ase (10).

Purification of the New Protein Factor. All operations were carried out at 4°. Deribbed spinach leaves (20 kg) were homogenized for 3 min in a Waring blendor in a solution of 50 mM Tris-HCl buffer (pH 7.6) and 0.1% 2-mercaptoethanol (hereafter called Buffer A) (1000 g of leaves and 1000 ml of Buffer A were used per blending.) (6). The extract was filtered through four layers of cheesecloth, and acetone, cooled to -15°, was added to a final concentration of 75%. The preparation was allowed to stand for 1 hr at -20°, the clear supernatant was decanted, and the precipitate was collected by centrifugation (2 min, 9,000 x *g*). The pellet was suspended in Buffer A to a final volume of 1.5 l and clarified by centrifugation (15 min, 40,000 x *g*).

The brown supernatant fraction was applied to a DEAE-cellulose column (5 x 50 cm) equilibrated beforehand with Buffer A and eluted with Buffer A supplemented with 0.3 M NaCl. The eluate was collected until the soluble ferredoxin had moved half-way down the column. The eluate was then fractionated with solid ammonium sulfate; the fraction precipitating between 30 and 90% saturation was collected by centrifugation (15 min, 9,000 x g) and dissolved in 300 ml of Buffer A. After clarification by centrifugation (30 min, 120,000 x g), the fraction was applied to a Sephadex G_{100} column (5 x 150 cm) equilibrated and developed with Buffer A. Fractions of 16 ml were collected and assayed for new protein factor and ferredoxin-thioredoxin reductase activities. The fractions containing the bulk of the new protein factor activity were combined and applied to a DEAE-cellulose column (2.5 x 30 cm) that was equilibrated beforehand with Buffer A supplemented with 0.06 M NaCl; the column was eluted with a linear gradient (1 liter) between 0.1 to 0.25 M NaCl in Buffer A. The fractions (5.5 ml) containing new protein factor activity were combined, dialyzed against Buffer A supplemented with 10 mM potassium phosphate buffer (pH 7.6), and then applied to a hydroxyapatite column (1.5 x 10 cm) that was equilibrated beforehand with Buffer A supplemented with 10 mM potassium phosphate buffer (pH 7.6). The hydroxyapatite column was then eluted separately with Buffer A solutions containing 50, 100, and 300 mM potassium phosphate buffer (pH 7.6). The fractions were concentrated by dialysis against solid sucrose (6) and then dialyzed against Buffer A until the dialyzate was free of P_i. The fraction that eluted with Buffer A supplemented with 300 mM P_i, which contained the bulk of the new protein factor activity, was then rechromatographed on a Sephadex G_{100} column (2.5 x 100 cm) equilibrated and developed with Buffer A. The fractions (2.5 ml) containing the new protein factor were concentrated by dialysis against solid sucrose to approximately 1 mg of protein per ml (determined after sucrose was removed by dialysis against Buffer A). This preparation served as the source of the new protein factor in the studies described below.

Preparation of Chloroplast Extract. Chloroplast extract was prepared from 800 g of spinach leaves as described previously (13) and was precipitated with solid ammonium sulfate (to 90% saturation). The precipitate was collected by centrifugation (15 min, 9,000 x g), resuspended in 20 ml of Buffer A, and clarified by a second centrifugation (30 min, 120,000 x g). The fraction was applied to a Sephadex G_{100} column that had been equilibrated and developed with Buffer A. Fractions of 3.8 ml were collected and assayed for the indicated activities.

RESULTS AND DISCUSSION

In recent studies on the purification of ferredoxin-thioredoxin reductase, we observed that certain column chromatography fractions catalyzed a photochemical activation of Fru-P_2ase when assayed with washed chloroplast membranes. Significantly, addition of individual components of the ferredoxin/thioredoxin system--ferredoxin, thioredoxin *f*, and ferredoxin-thioredoxin reductase--had little effect on Fru-P_2ase activation under these conditions. On examination, the active factor in these fractions showed the properties of a protein--i.e., it was nondialyzable and was sensitive to trypsin and to heat [25% and 5% of initial activity remaining, respectively, after 5 min of trypsin digestion (14) and 3 min of heating at 80°]. Consequently, the factor was, pending a more complete description of its properties, designated the "new protein factor."

The new protein factor was present in the soluble fraction of isolated chloroplasts (chloroplast extract), and, as shown in Fig. 1, could be separated from Fru-P_2ase and soluble ferredoxin by Sephadex G_{100} chromatography. The new protein factor was also partly separated by the G_{100} treatment from both thioredoxin f (the thioredoxin specific for Fru-P_2ase) and ferredoxin-thioredoxin reduct

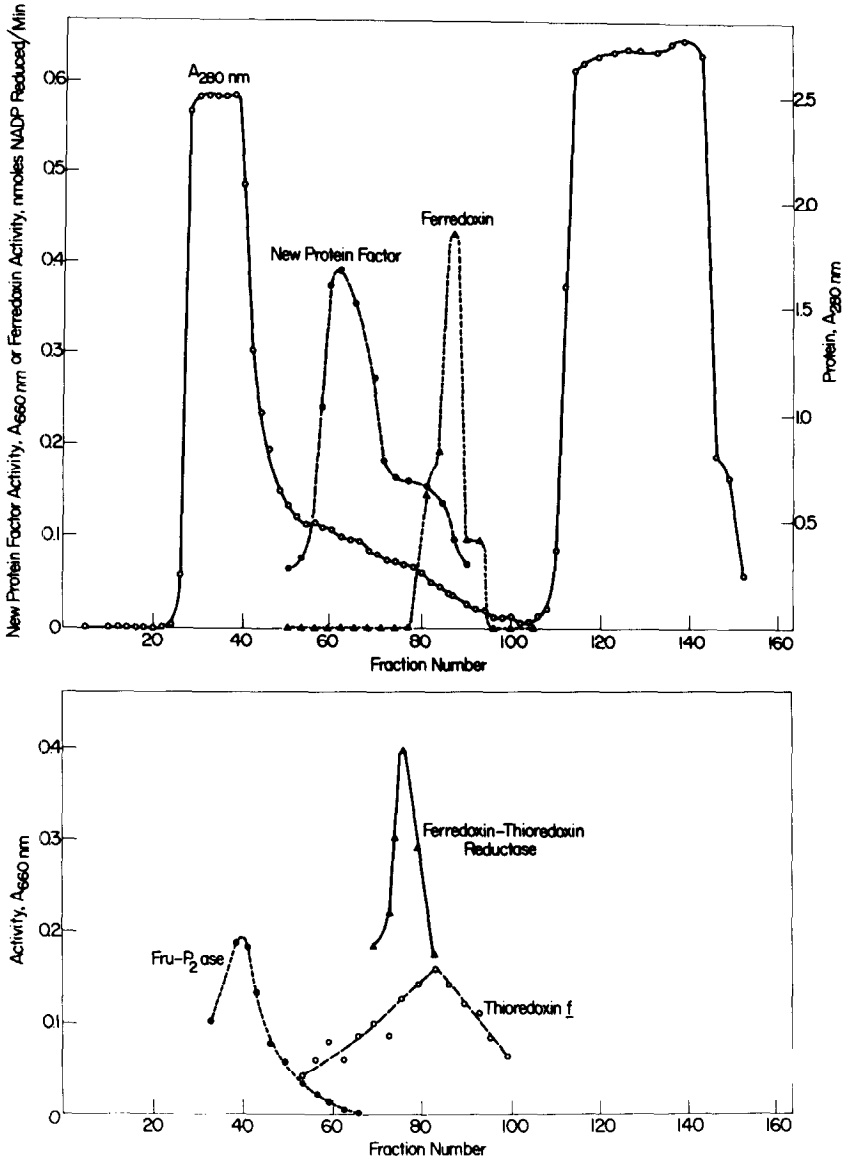

Figure 1. Demonstration of the new protein factor in chloroplast extract after Sephadex G_{100} chromatography. The protein and activity profiles indicated in both the upper and lower panels were determined in a single column eluate that was obtained as given in METHODS.

ase (Fig. 1). Parallel experiments revealed that, as with other chloroplast proteins, the activity of the new protein factor was, on a protein basis, lower in leaf extracts than in chloroplast extracts. Despite this concentration difference, it proved convenient to utilize leaves as the starting material for large-scale preparations.

With the leaf preparations, we found, as with chloroplasts, that Sephadex G_{100} chromatography partially separated the new protein factor from ferredoxin-thioredoxin reductase (Fig. 2). Removal of the remaining ferredoxin-thioredoxin reductase from most preparations was difficult even with extensive purification. It is significant that some preparations of the new protein factor were totally freed of ferredoxin-thioredoxin reductase activity when the initial G_{100} chromatography step was followed, as described in Methods, by sequential chromatography on DEAE-cellulose, hydroxyapatite, and Sephadex G_{100} columns. Similarly, ferredoxin-thioredoxin reductase purified by the recently described method (6,8) showed no activity indicative of the new protein factor. It is thus clear that, despite

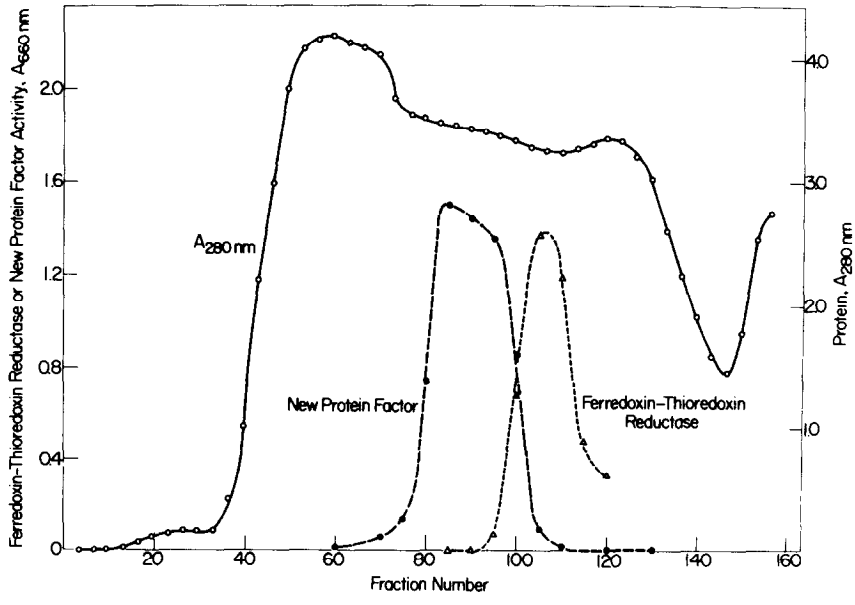

Figure 2. Separation of new protein factor from ferredoxin-thioredoxin reductase in leaf extract by Sephadex G_{100} chromatography.

their close association during purification, these two activities reside on different proteins. Highly purified preparations of the new protein factor consistently showed no ferredoxin, thioredoxin f, or thioredoxin m activity. Determination of the chemical properties of the new protein factor is in progress.

The requirements of the new protein factor for the light activation of Fru-P_2ase were simple. Highly purified preparations of the new protein factor consistently activated Fru-P_2ase in a reaction that depended only on light and chloroplast membranes that were shown to be ferredoxin-free (11) (Table 1). The components essential for the activity of Fru-P_2ase itself--i.e., Mg^{++} and fructose 1,6-bisphosphate--were also required for P_i release under these conditions. Identical requirements were observed when chloroplasts were replaced by chlorophyllous membrane fragments purified from the blue-green alga, Nostoc muscorum, even after passage through a DEAE-cellulose column to insure removal of possible traces of soluble ferredoxin and of ferredoxin-NADP reductase.

Fru-P_2ase that was activated photochemically by the new protein factor was deactivated by dehydroascorbate and oxidized glutathione--oxidants shown earlier to deactivate thioredoxin-activated Fru-P_2ase (4,14). (In each case, 35% of the control activity remained after 7 mM oxidant was added to the activated Fru-P_2ase

Table 1. Requirements for Fru-P_2ase activated photochemically with a purified preparation of the new protein factor

	Fru-P_2ase Activity (nmol of P_i released per min)
Light	
Complete	136
Minus new protein factor	0
" Mg^{++}	0
" fructose 1,6-bisphosphate	0
" Fru-P_2ase	0
" chloroplast membranes	0
Dark	
Complete	0

as given in ref. 14.) It thus appears that the mode of activation of Fru-P_2ase by the new protein factor is similar to that of thioredoxin.

CONCLUDING REMARKS

The present results provide evidence that the light-actuated regulation of Fru-P_2ase in chloroplasts can be effected by a ferredoxin-independent mechanism in addition to the earlier-described ferredoxin-linked thioredoxin system. These findings prompt a recollection of the idea, originally expressed by Anderson and Avron (15), that chloroplasts photochemically generate membrane-bound reductants that function in enzyme regulation without the intervention of ferredoxin or other soluble factors. While supporting the general concept of a protein-mediated regulatory mechanism that is independent of ferredoxin, our results speak against the aspect of the Anderson/Avron proposal pertaining to soluble factors. We find that a soluble protein indigenous to chloroplasts (the new protein factor) is consistently required for the ferredoxin-independent light activation of Fru-P_2ase. Apropos this point, it is noteworthy that the recent results of Ashton and Anderson (16,17) also indicate that an unidentified soluble protein is needed for the light modulation of enzymes in a pea chloroplast system.

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