ISOLATION OF TWO PROTEINS WITH CHLOROPLAST FERREDOXIN ACTIVITY FROM A BLUE-GREEN ALGA

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The importance of ferredoxins as components of several electron transfer pathways in bacteria and plants has been firmly established. The name "ferredoxin" was originally given to a non-heme, iron-containing protein which functions as an electron-transfer factor in Clostridium pasteurianum (Mortenson et al., 1962). Tagawa and Arnon (1962) have extended the use of this term to include proteins with related chemical and biological properties (e.g. photosynthetic pyridine nucleotide reductase) that had previously been found in chloroplasts (San Pietro and Lang, 1958; Davenport, 1960). The experiments of San Pietro and Lang (1958) showed that chloroplast ferredoxin functions in a light-dependent electron transfer pathway in isolated chloroplasts terminating in the reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$).

The requirement of chloroplast ferredoxin for the photoreduction of $NADP^+$ by isolated chloroplasts is not highly specific since C. pasteurianum

ferredoxin or benzyl viologen may be substituted (Tagawa and Arnon, 1962). Nevertheless, chloroplast ferredoxin is thought to be an essential and specific component of the pathway for the photoreduction of $NADP^+$ in intact chloroplasts. During an investigation of the roles in photosynthesis of several proteins extracted from blue-green algae, two proteins catalyzing

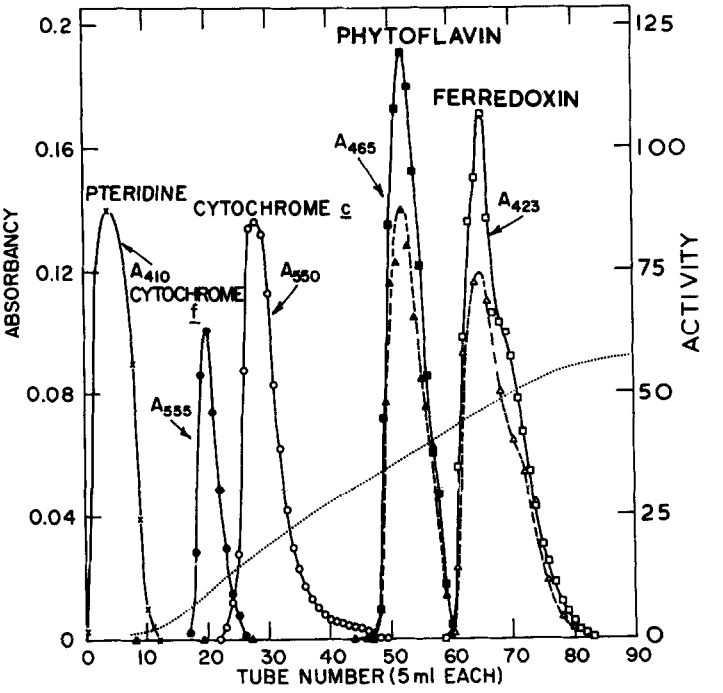


Fig. 1. Separation of two proteins with chloroplast ferredoxin activity by DEAE-cellulose column chromatography.

Washed cells of A. nidulans (100 g wet weight) were suspended in 0.05 M tris buffer, pH 7.8 and were broken by passage through a French Pressure Cell at 20,000 p.s.i. The mixture was centrifuged at 144,000 g for 60 min and the supernatant fluid was fractionated with ammonium sulfate. The 65-100% saturated ammonium sulfate fraction was dissolved in 0.05 M tris pH 7.8, dialyzed against 0.005 M tris buffer pH 7.8, and absorbed on a DEAE-cellulose column. Protein was eluted by an increasing non-linear gradient of NaCl. A mixing vessel contained 400 ml of 0.005 M tris buffer pH 7.8 and a reservoir contained 0.6 M NaCl in 0.005 M tris buffer pH 7.8. The continuous line in the figure shows the absorbancy of individual fractions at the wave-lengths indicated. The dashed line and triangles show chloroplast ferredoxin activities in mμmoles of $NADP^+$ reduced per minute per ml fraction. The dotted line indicates the salt gradient (conductivity measurements). Chloroplast ferredoxin activity was assayed as described previously (Smillie, 1962). The reaction mixture contained washed chloroplasts (18 μg chlorophyll) isolated from pea leaves, tris-HCl buffer pH 7.8 (20 mM), $MgCl_2$ (2.5 mM) and $NADP^+$ (110 μM). The final volume was 0.8 ml and the assay temperature was 25°C. The activity and absorbancy values shown for fractions containing ferredoxin are one-fifth the actual values obtained.

the photoreduction of $NADP^+$ by chloroplasts were isolated. One of these proteins is not a ferredoxin. It has been named phytoflavin.

RESULTS

Fig. 1 shows a column chromatographic separation on DEAE cellulose of colored proteins from a cell-free extract of the blue-green alga Anacystis nidulans. Assays for chloroplast ferredoxin activity (i.e. the capacity to catalyze the reduction of $NADP^+$ by illuminated chloroplasts) disclosed two distinct peaks of activity (designated as phytoflavin and ferredoxin). The distribution of activity in each peak coincided with a colored component.

Figs. 2 and 3 show the absorption spectra of the two proteins showing chloroplast ferredoxin activity.

The absorption spectrum of phytoflavin (Fig. 2) is characteristic of a flavoprotein and not of a ferredoxin. The colored component of phytoflavin was split from the protein by heat, acidification to pH 4, or digestion with

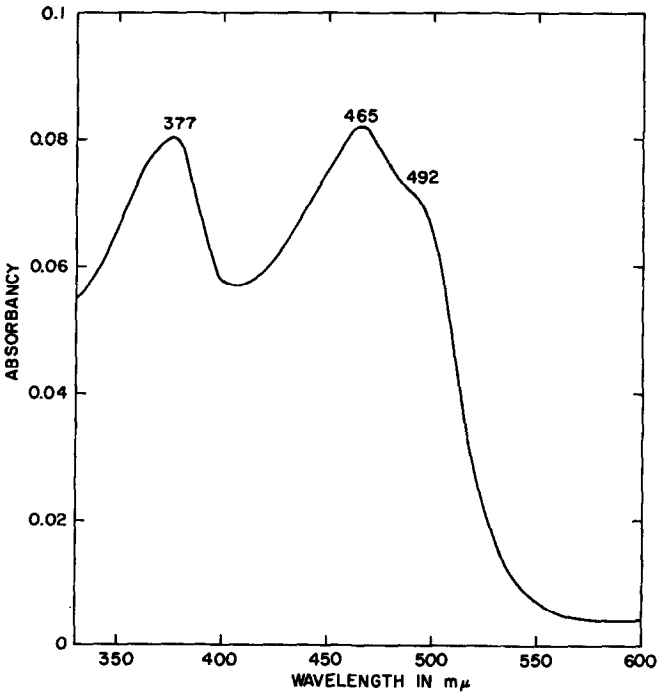


Figure 2. Absorption spectrum of phytoflavin.

proteolytic enzymes. It was identified as flavin adenine mononucleotide (FMN) by paper chromatography and by bioassay with apo-cytochrome c reductase. Flavin adenine dinucleotide (FAD) was not detected.

The other active protein appeared to be analogous to chloroplast ferredoxin. Its absorption spectrum (Fig. 3) corresponded with that of a chloroplast ferredoxin isolated from parsley leaves by Davenport (see Fry and San Pietro, 1963). Chloroplast ferredoxin prepared from either leaves (pea and turnip) or green algae (Chlamydomonas and Euglena) behaved similarly to the A. nidulans ferredoxin on a DEAE-cellulose column. The existence of a chloroplast-type ferredoxin in A. nidulans has already been demonstrated by Black et al. (1963a).

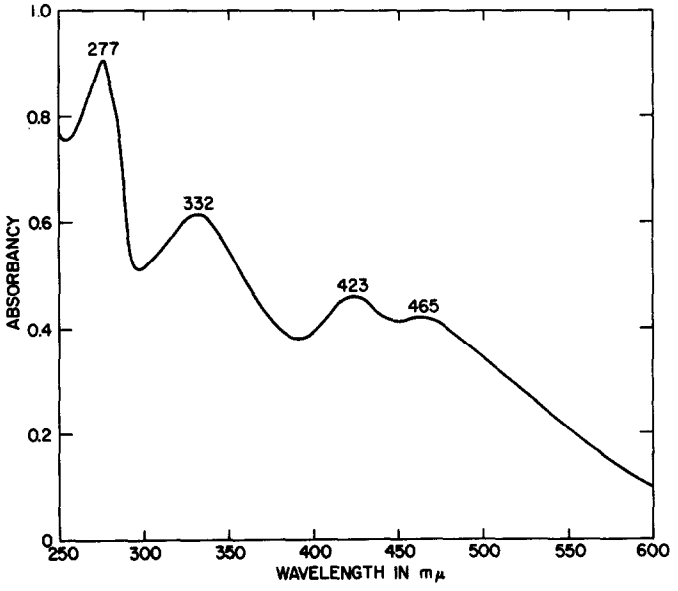


Figure 3. Absorption spectrum of the chloroplast-type ferredoxin.

In Table 1 the phytoflavin and the chloroplast-type ferredoxin from A. nidulans are compared for their capacity to catalyze the photoreduction of $NADP^+$ by washed isolated chloroplasts. An assay system containing pea leaf chloroplasts was used in this experiment, but similar results were obtained with chloroplasts isolated from Euglena gracilis or from leaves

of wheat or spinach. Heated flavoprotein (100°C for 4 min) or FMN was inactive in this system.

TABLE 1

Activity of the Protein Fractions from A. nidulans in the Photoreduction of $NADP^+$ by Chloroplasts

Protein fraction	mμmole added in assay	Rate of $NADP^+$ Reduction	
		mμmoles $NADP^+$/ min in assay	moles $NADP^+$/min /mole ferredoxin or FMN in Phytoflavin
No additions	-	0	-
Phytoflavin	0.38	3.71	9.80
Heated phytoflavin	0.38	0	-
Ferredoxin	0.59	2.59	4.38
Heated ferredoxin	0.59	0	-

The reaction mixture is given in Fig. 1. The concentrations of phytoflavin and ferredoxin were determined spectrophotometrically.

The chloroplast ferredoxin activity of phytoflavin cannot be attributed to contamination by either chloroplast- or bacterial-type ferredoxins. The activity of phytoflavin (per mole FMN) in promoting the photoreduction of $NADP^+$ was higher than that of ferredoxin (Table 1). The activity of bacterial ferredoxin in this reaction is considerably lower than either (see Davenport, 1963). If the activity were due to contamination by a chloroplast-type ferredoxin, the latter would have contributed significantly to the absorption spectrum of the phytoflavin since the extinction coefficients of FMN (ϵ_{450} = 12.2 cm^{-1} mM^{-1}; Cerletti, 1959) and chloroplast ferredoxin (ϵ_{420} = 10.32 cm^{-1} mM^{-1}; Whatley et al., 1963) are similar. The relatively low absorption of phytoflavin above 550 mμ (Fig. 2) indicated the absence of significant amounts of non-heme iron of the type found in ferredoxins or

iron-flavoproteins such as xanthine oxidase (Handler et al., 1964). An additional indication that the activity of phytoflavin was not due to a ferredoxin component was obtained in an experiment in which cells were grown in a medium containing Fe^{55}. The purification procedure described in Fig. 1 was repeated using these cells. DEAE-cellulose column chromatography revealed a peak of gamma-emitting activity which coincided with the ferredoxin peak. Fractions containing phytoflavin showed only trace amounts of radioactivity.

One other flavoprotein has been shown to function in the photoreduction of $NADP^+$ by isolated chloroplasts. This protein, pyridine nucleotide transhydrogenase, was purified from spinach by Keister et al. (1960). It has a similar absorption spectrum to that of phytoflavin and contains FAD. It exhibits $NADP^+$-reductase activity and is thought to be the terminal enzyme in the pathway of $NADP^+$ photoreduction (Shin et al., 1963). The biological activity of phytoflavin is quite different from the $NADP^+$-reductase of chloroplasts. The reductase will not support the photoreduction of $NADP^+$ by isolated chloroplasts in the absence of ferredoxin. Chloroplast $NADP^+$-reductases have been isolated from several different plant sources and all show NADPH-diaphorase activity (Davenport, 1963). Most also exhibit pyridine nucleotide transhydrogenase activity. Phytoflavin, on the other hand, did not react directly with pyridine nucleotide (Table 2). It showed neither dehydrogenase nor oxidase activity when tested with a wide range of possible substrates and acceptors.

A comparison of the rates of photoreduction of NAD^+, $NADP^+$ and the 3-acetylpyridine analog of $NADP^+$ by isolated chloroplasts supplemented with phytoflavin indicated that the phytoflavin, like ferredoxin, reduced pyridine nucleotides via $NADP^+$-reductase. The reverse reaction, i.e. the reduction of phytoflavin by NADPH catalyzed by $NADP^+$-reductase, was demonstrated spectrophotometrically. The addition of NADPH to phytoflavin and a catalytic amount of purified chloroplast $NADP^+$-reductase resulted in the reduction of the phytoflavin as indicated by the disappearance of the

TABLE 2

NADPH-diaphorase and Transhydrogenase Assays of the

A. nidulans proteins and Chloroplast $NADP^+$-reductase

Protein	μμmoles ferredoxin or flavin in assay	NADPH-diaphorase	Transhydrogenase
		mμmoles NADPH oxidized/min	
Phytoflavin	380	0.2	0
Ferredoxin	590	0	0
$NADP^+$-reductase (from spinach)	2.5	6.8	3.5

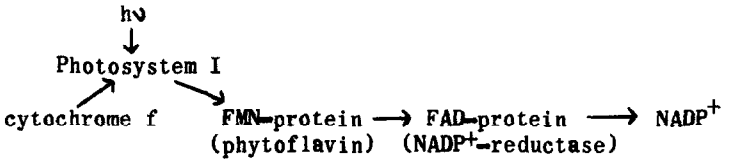
NADPH-diaphorase was assayed according to Avron and Jagendorf (1956) and transhydrogenase according to Keister et al. (1960). The $NADP^+$-reductase was purified from spinach leaves using the procedure of Keister et al. (1960).

465 mμ and 492 mμ absorption peaks. The appearance of a new absorption maximum at 600 mμ indicated that reduction did not proceed beyond the semiquinone form (Massey and Gibson, 1964).

DISCUSSION

Chloroplast ferredoxin is thought to be an essential component of the pathway of $NADP^+$ photoreduction in chloroplasts. This conclusion is based on experiments in which it was shown that washed preparations of isolated chloroplasts cannot photoreduce $NADP^+$ unless supplemented with chloroplast ferredoxin. The isolation of a new protein is now reported that will replace chloroplast ferredoxin in mediating the photoreduction of $NADP^+$ by chloroplasts. The new protein is not a ferredoxin and contains FMN. Its activity is comparable to that of chloroplast ferredoxin (mole FMN in phytoflavin to mole ferredoxin). Phytoflavin occurs in Anacystis nidulans and Anabaena cylindrica, but other organisms have not been examined.

In separate experiments (Smillie, 1964), it has been shown that isolated chloroplasts from Euglena gracilis photo-oxidize cytochrome f. This oxidation is dependent on the presence of either ferredoxin or phytoflavin. It is suggested that phytoflavin can mediate in the photoreduction of $NADP^+$ by isolated chloroplasts by means of a coupled reaction with cytochrome f. Light absorbed by photosystem I of the chloroplasts results in the photo-oxidation of cytochrome f coupled to the reduction of phytoflavin. The latter in turn reduces $NADP^+$ via a FAD-protein (chloroplast $NADP^+$-reductase) as illustrated in the following reaction sequence:



The question arises why A. nidulans contains two dissimilar proteins with apparently similar biological activities. Tagawa et al. (1963) have shown that ferredoxin can act as an electron carrier for cyclic photophosphorylation in isolated chloroplasts. The role of phytoflavin may be restricted to the non-cyclic system, thus allowing for an additional control mechanism for determining the main pathway of electron flow. These considerations are complicated by the recent discovery of phosphodoxin (Black et al. 1963b), another naturally occurring substance which functions as a cofactor for cyclic photophosphorylation.

Another possible function for phytoflavin is that it might react specifically with enzymes that result in the reduction of metabolites other than $NADP^+$ such as NAD^+ and nitrate.

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