BBA 46256

FERREDOXINS IN LIGHT- AND DARK-GROWN PHOTOSYNTHETIC CELLS WITH SPECIAL REFERENCE TO *RHODOSPIRILLUM RUBRUM*

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(Received August 3rd, 1971)

SUMMARY

Two different ferredoxins (type I and type II) were isolated from photosynthetically grown *Rhodospirillum rubrum* cells. Type I had 6 non-heme irons and 6 acid-labile sulfides and an absorption ratio $A_{385nm}/A_{280nm} = 0.72$; type II had 2 non-heme irons and 2 acid-labile sulfides and an absorption ratio $A_{385nm}/A_{280nm} = 0.52$. The ferredoxins differed in amino acid composition and molecular weight (type I, 8700; type II, 7500). *R. rubrum* cells grown heterotrophically in the dark yielded type II ferredoxin; type I ferredoxin accompanied type II only when the cells were grown in the light. By comparison, spinach seedlings had only one type of ferredoxin whether germinated in the light or in the dark.

R. rubrum appears to be the first organism from which two types of ferredoxin (one formed only in the light) were isolated. *R. rubrum* ferredoxin type II is the first instance of a ferredoxin from a photosynthetic bacterium that has a bacterial type absorption spectrum but a non-heme iron and labile sulfide content (two of each per mole) that is characteristic of plant ferredoxins.

INTRODUCTION

The iron-sulfur proteins known as ferredoxins have been shown to play a major role in plant and bacterial photosynthesis[1-7]. The importance of ferredoxins in photosynthesis raises the question whether they are constitutive proteins in photosynthetic cells and are present even when these are grown heterotrophically without light. This question was deemed particularly pertinent to an organism such as *Rhodospirillum rubrum* which is noted among photosynthetic bacteria for its ability to grow either anaerobically under photosynthetic conditions or aerobically without light. Light- and dark-grown spinach seedlings were used to assay the formation of plant-type ferredoxin.

An unexpected finding was that *R. rubrum* produces two types of ferredoxin, one type formed only in the light and a second type formed when the cells are grown either in the light or in the dark. In spinach, the same type of ferredoxin was found both in the light- and in the dark-grown seedlings.

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

METHODS

Culture methods

R. rubrum S-1 cells (obtained from Dr. L. R. Berger of the University of Hawaii) were grown in the malate–ammonia medium of ORMEROD *et al.*[8] anaerobically in the light for 3 to 5 days from a 10 to 15 % (by vol.) inoculum, in 13-l carboys illuminated with flood lamps as described by ARNON *et al.*[9].

The dark cultures were placed in 13-l carboys covered with aluminum foil and grown (with vigorous and continuous bubbling of air) by adding 1 l of inocolum to 9 l of the malate–ammonia[8] medium. The cells were harvested after 48 h of growth in the dark.

Spinach seeds (var. Resistoflay) were germinated in sand, in the dark, in a solution containing KH_2PO_4, $Ca(NO_3)_2$, and $MgSO_4$, each at 0.1 mM. After 8 days, half of the seedlings were exposed for 3 days to daylight (supplemented with continuous fluorescent light). The shoots of seedlings exposed to light and of those kept for the entire 11 days in the dark were cut off at the sand level and stored at $-20°$. Light- and dark-grown barley (var. California Marion) seedlings were obtained in the same way, except that the seedlings were grown for a total of only 7 days. Half of them were removed from the darkroom after 4 days and exposed to light for 3 days prior to harvest.

Isolation of ferredoxins

Rhodospirillum ferredoxins. The sequence followed in the isolation and purification of Rhodospirillum ferredoxins included sonication of cells, treatment with the non-ionic detergent Triton X-100, adsorption and purification by DEAE-cellulose chromatography, and separation of two types of ferredoxin by Sephadex G-100 column chromatography. Rapid purification was important for minimizing denaturation of Rhodospirillum ferredoxins which were sensitive to air.

The cells were suspended in 1.5 vol. of 0.05 M potassium phosphate buffer (pH 7.3). The suspension was sonicated for 5 min at 4° using a 20 KC Branson sonifier at an output power of 8.5 A. Triton X-100 was added to the sonicate to a final concentration of 5 % and the mixture was incubated in the cold for 30 min with constant stirring. Pre-cooled acetone (−20°) was then added to a final concentration of 30 % (by vol.) and the mixture was incubated, with stirring, for an additional 30 min in the cold and centrifuged at 13000 × *g* for 20 min. The residue was discarded and the supernatant fluid (containing acetone) was centrifuged at 30000 × *g* for 15 min to remove the small particulate material that interfered in the DEAE-cellulose chromatography step.

The clear, purple, supernatant fluid (containing acetone) was passed through a 5 cm × 15-cm DEAE-cellulose column equilibrated with 0.02 M potassium phosphate buffer (pH 7.3). The ferredoxin was adsorbed at the top of the column. The column was washed at a flow rate of about 700 ml/h with 500 ml of 0.02 M potassium phosphate buffer (pH 7.3), followed by another 500 ml of 0.01 M potassium phosphate buffer (pH 7.3) containing 0.2 M NaCl. The adsorbed crude ferredoxin was eluted with 0.01 M potassium phosphate buffer (pH 7.3) containing 0.8 M NaCl. [Thereafter, the buffer used to equilibrate all DEAE-cellulose columns was 0.02 M potassium phosphate (pH 7.3); the columns were also washed or eluted with this buffer at half strength (0.01 M but containing the indicated concentrations of NaCl)].

The reddish eluate containing crude ferredoxin was diluted with ice-cold distilled water to give a NaCl concentration of 0.1 M and adsorbed on a second DEAE-cellulose column (3 cm × 15 cm). This step separated ferredoxin from a contaminating red material; ferredoxin, recognized by its brown color, was adsorbed at the top of the column above the red band. The column was washed with 500 ml of buffer containing 0.2 M NaCl. Ferredoxin was then eluted with buffer containing 0.8 M NaCl. The red band remained on the column.

Ice-cold distilled water was added to the eluate to bring the NaCl concentration down to 0.1 M and the diluted eluate was placed on a third DEAE-cellulose column (3 cm × 10 cm) for partial purification. The column was first washed with 500 ml of buffer containing 0.2 M NaCl. Washing with buffer containing 0.26 M NaCl started the movement of the ferredoxin band; when it reached the middle of the column it was eluted by increasing the concentration of NaCl in the buffer to 0.3 M. The partly purified ferredoxin was collected, in a volume of about 500–600 ml.

After adding distilled water to lower the concentration of NaCl to 0.1 M, the collected ferredoxin was concentrated by adsorption on a short DEAE-cellulose column (2 cm × 3 cm) and elution with buffer containing 0.8 M NaCl (volume of eluate was 10–15 ml).

The concentrated ferredoxin solution (diluted to lower the NaCl concentration to 0.1 M) was chromatographed on a fourth 2.5 cm × 45-cm DEAE-cellulose column and eluted with buffer containing 0.3 M NaCl. The eluted ferredoxin was concentrated as before, using a short DEAE-cellulose column, to a volume of about 3 ml.

The concentrated ferredoxin solution was chromatographed on a Sephadex G-100 column (2 cm × 60 cm, superfine grade) using 0.05 M potassium phosphate buffer (pH 7.3) as eluent. Two brown bands of ferredoxin were observed at this stage and each was collected separately. The Sephadex chromatography was also effective in removing contaminating nucleic acids.

Isolation of spinach and barley ferredoxin. The seedling shoots were placed in 0.05 M phosphate buffer, pH 7.3 (volume equal to twice the weight of shoots) and disrupted by blending in a Waring blendor for 3 min. The slurry was filtered through four layers of cheesecloth, mixed slowly with an equal volume of cold acetone (−20°), and stirred in the cold for 30 min. The mixture was centrifuged for 15 min at 13000 × *g*. The residue was discarded and the supernatant fluid passed through a DEAE-cellulose column (3 cm × 15 cm), equilibrated beforehand with 0.02 M potassium phosphate buffer (pH 7.3). The column was washed successively with 500-ml portions each of 0.02 M phosphate buffer (pH 7.3) and 0.01 M phosphate buffer (pH 7.3) containing 0.2 M NaCl. The ferredoxin was eluted with the same buffer containing 0.8 M NaCl.

After adding distilled water to lower the concentration of NaCl to 0.1 M, the eluted ferredoxin was concentrated, as before, by adsorption on, and elution (in a volume not exceeding 10 ml) from, a small column of DEAE-cellulose. The concentrated ferredoxin solution was (prior to use) dialyzed overnight against 0.05 M Tris–HCl buffer (pH 8.0). Ferredoxin from barley was isolated in the same way except that the acetone step was omitted and the initial extract was dialyzed overnight against 10 vol. of buffer before passing through the DEAE-cellulose column. The chlorophyll content of spinach and barley seedlings was estimated as described by ARNON[10].

Other procedures. Non-heme iron and acid-labile sulfide were estimated as de-

scribed by LOVENBERG *et al.*[11]. The biological activity of ferredoxin was measured by its ability to replace spinach ferredoxin in $NADP^+$ reduction by washed, broken spinach chloroplasts as described by MCSWAIN AND ARNON[12]. To estimate the concentration of Rhodospirillum ferredoxins their molar extinction coefficients were determined at 390 nm. Gel electrophoresis was carried out as described by DAVIS[13]. Absorption spectra were recorded in a Cary Model-14 recording spectrophotometer.

The bacteriochlorophyll content of the *R. rubrum* cells was estimated as described by COHEN-BAZIRE *et al.*[14].

RESULTS AND DISCUSSION

Ferredoxins I and II from photosynthetically grown Rhodospirillum cells

Chemical and physical properties. The ferredoxin preparation obtained from the *R. rubrum* cells grown in the light gave a single peak in DEAE-cellulose chromatography and one band in polyacrylamide gel electrophoresis. However, when this preparation was chromatographed on a Sephadex G-100 column, two peaks of ferredoxin were observed (Fig. 1). The slower moving ferredoxin peak was designated as type I and the faster one, as type II. The ratio of type I to type II was about 0.45:0.55.

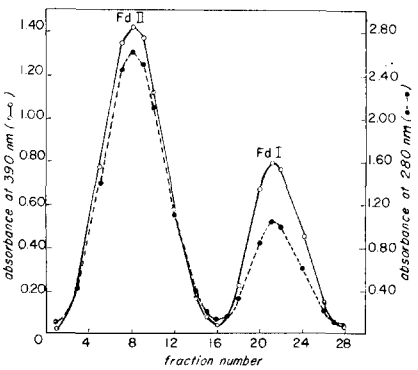

Fig. 1. Chromatography of Rhodospirillum ferredoxins on Sephadex G-100. Collection of 1.5-ml fractions began just prior to the elution of the brown ferredoxin fraction from the column.

Type I showed a broad absorption maximum at 385 nm, a minimum at 350 nm, and a peak at 258 nm with a shoulder at 300 nm (Fig. 2). Type II showed a broad absorption band centered at 385 nm, with no minimum at 350 nm, and a pronounced absorption peak at 280 nm. Because of the difference in absorption at 280 nm, type I had an $A_{385\,nm}/A_{280\,nm}$ ratio of 0.72, compared with a ratio of 0.52 for type II. The spectrum of type II was stable but that of type I underwent change with time: immediately after elution from the column it had an $A_{385\,nm}/A_{280\,nm}$ ratio as high as 0.8 which decreased on storage of the ferredoxin. In this respect, type I resembles Chlorobium ferredoxin[15].

Minimum molecular weights calculated from the amino acid composition (discussed later) and the non-heme iron and labile sulfide content came to 8700 for type I and 7500 for type II (Tables I and II). Based on the minimum molecular weights, the extinction coefficients (385 nm) for types I and II were calculated, respectively,

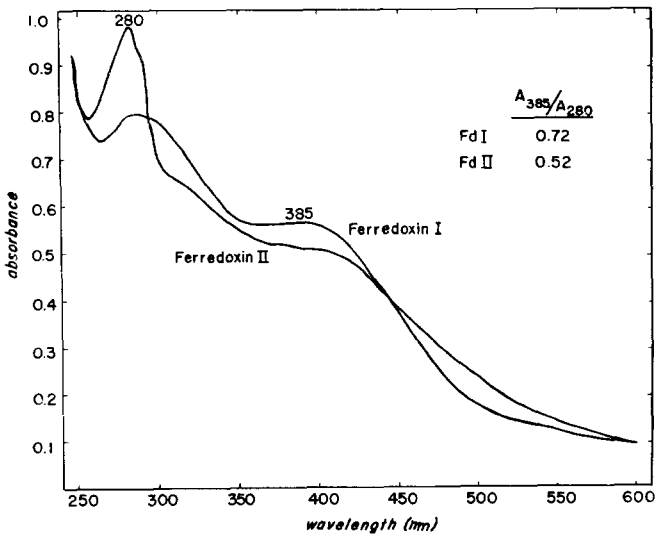

Fig. 2. Absorption spectra of Rhodospirillum ferredoxins. Ferredoxin type I (0.2 mg/ml) and ferredoxin type II (0.45 mg/ml) in 0.05 M potassium phosphate buffer (pH 7.3); light path, 1 cm.

TABLE I

MOLAR CONTENT OF NON-HEME IRON AND ACID-LABILE SULFIDE IN DIFFERENT FERREDOXINS

Source of ferredoxin	*Non-heme iron*	*Acid-labile sulfide*
R. rubrum (I)	6	6
R. rubrum (II)	2	2
Spinach chloroplasts (ref. 2)	2	2
Chromatium D*	8	8
*Chlorobium thiosulfatophilum***	8	8
Clostridium pasteurianum (ref. 16)	8	8

* Based on a molar extinction coefficient of 31000 (ref. 19) *Chromatium* ferredoxin now appears to have 8 non-heme iron and acid-labile sulfide groups per mole.

** Based on protein estimation, the reported value for non-heme iron and acid-labile sulfide content of Chlorobium ferredoxin was 5 (ref. 15). However, a value of 8 is obtained by recalculation, based on the assumption that the molar extinction coefficient of Chlorobium ferredoxin is close to that of clostridial ferredoxin[16].

as 24.3 and 8.8 $mM^{-1} \cdot cm^{-1}$ (compared to 30.6 for clostridial ferredoxin at 390 nm[16] and 9.7 for spinach ferredoxin at 420 nm[17]).

Type I ferredoxin and type II ferredoxin differed in their content of non-heme iron and acid-labile sulfide (Table I). Type I, with 6 irons and sulfides, resembled other bacterial ferredoxins but type II — unexpectedly for a bacterial ferredoxin[2,3] — was found to have only two non-heme iron and labile sulfide groups that are characteristic of plant ferredoxins. Despite this similarity to plant ferredoxins, the absorption spectrum of type II ferredoxin, with its one broad peak at 385 nm (Fig. 2)

TABLE II

AMINO ACID COMPOSITION OF TYPES I AND II OF *R. rubrum* FERREDOXIN IN COMPARISON WITH OTHER FERREDOXINS

Amino acid residue	*R. rubrum*		*Spinach*[18]	*Chromatium*[19]	*Chlorobium*[15]	*Clostridium pasteurianum*[23]
	Type I	*Type II*				
Lys	4	5	4	2	0	1
His	1	0	1	2	0	0
Arg	0–1	1	1	2	0	0
Try	0	2	1	0	0	0
Asp*	9	10	13	8	3	8
Thr	4	2–3	8	6	4	1
Ser	3	2	7	4	2	5
Glu*	12	8	13	16	7–8	4
Pro	4–5	4	4	5	4	3
Gly	4	4	6	5	4–5	4
Ala	7	6	9	3	11	8
Cys	6	3	5	9	7–8	8
Val	7	6	7	6	3	6
Met	0–1	0–1	0	1	0	0
Ileu	6	4	4	6	4	5
Leu	3	4	8	3	1	0
Tyr	0	1	4	3	2	1
Phe	3	2–3	2	0	1	1
Total	73–76	65–67	97	81	53–56	55

* No distinction was made between aspartate and asparagine or glutamate and glutamine.

resembled that of other bacterial ferredoxins: it lacked the absorption maxima at 463, 420, and 325 nm characteristic of plant ferredoxins[1–3].

Type I ferredoxin was readily reduced by sodium dithionite, whereas type II ferredoxin was reduced by dithionite only in the presence of methyl viologen (Fig. 3). Methyl viologen increased slightly the extent of reduction of type I. After reduction, both ferredoxins were reoxidized completely by air. The two ferredoxins were also reduced by NADPH through a reversal of the ferredoxin–$NADP^+$ reductase system from spinach[20].

Table II gives the amino acid composition of the two Rhodospirillum ferredoxins and a comparison with those of Chlorobium, *Chromatium*, spinach, and clostridial ferredoxins. Like other ferredoxins, both Rhodospirillum ferredoxins have a large amount of acidic amino acids, *i.e.* aspartate and glutamate (about 27 % of the total). Despite the similarities, the two Rhodospirillum ferredoxins differed in certain amino acids. Type I, but not type II, had one histidine per molecule. Type II, but not type I, had two tryptophan and one tyrosine residues per molecule. Tryptophan had not been found previously in ferredoxins isolated from an anaerobic organism[3].

Substitution for spinach ferredoxin. As shown in Fig. 4, both Rhodospirillum ferredoxins were capable of replacing spinach ferredoxin in mediating electron transfer from illuminated chloroplasts to $NADP^+$, in the presence of chloroplast ferredoxin–$NADP^+$ reductase[20]. In mediating the photoreduction of $NADP^+$, type I was, at lower concentrations, indistinguishable from spinach ferredoxin; type II was less active (Fig. 5). Both ferredoxins were capable of replacing Chlorobium ferredoxin in

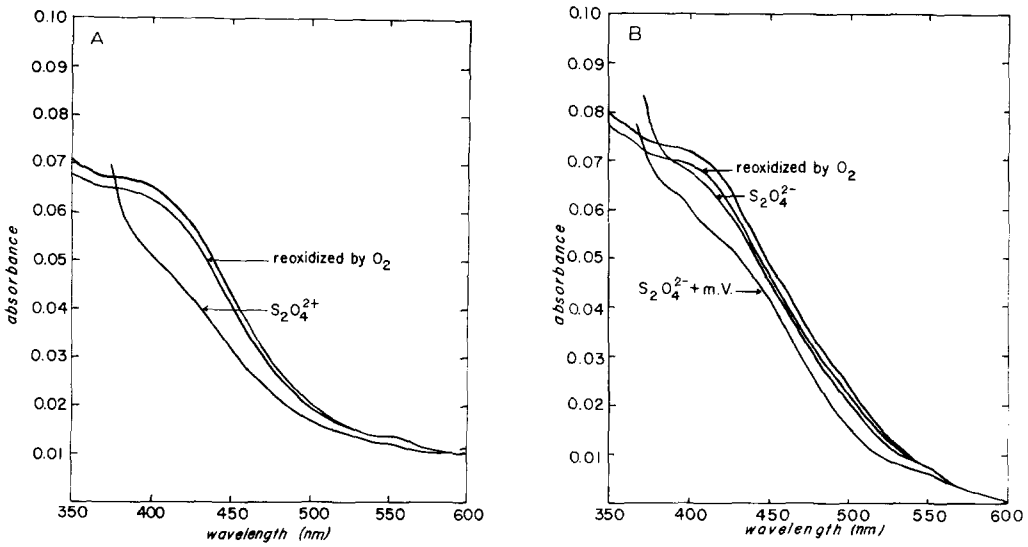

Fig. 3. Reduction and reoxidation of Rhodospirillum ferredoxins. (A) Type I. (B) Type II. The ferredoxins were reduced by adding a few crystals of sodium dithionite and were reoxidized by bubbling oxygen gas. Methyl viologen (m.V.; B) was added at a final concentration of $4 \cdot 10^{-7}$ M. Ferredoxin type I (0.12 mg/ml) and type II (0.3 mg/ml) in 0.05 M potassium phosphate buffer (pH 7.3); light path, 0.2 cm.

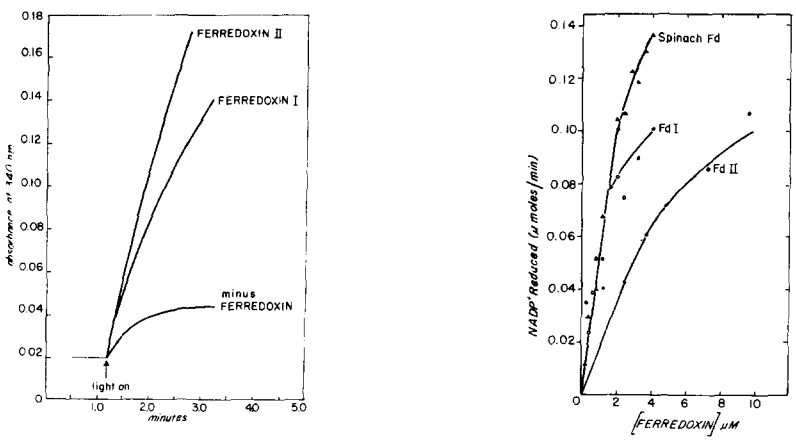

Fig. 4. Activity of Rhodospirillum ferredoxins in $NADP^+$ reduction by washed spinach chloroplasts. The reaction mixture, in 1 ml, contained: Tris–HCl buffer (pH 8.0), 50 mM; ascorbate, 10 mM; DCIP, 0.1 mM; NADP, 1 mM; washed chloroplasts, 100 μg chlorophyll and ferredoxin. Ferredoxin type I, 0.4 μM; ferredoxin type II, 2.4 μM. Light path, 0.2 cm.

Fig. 5. Effect of concentration of Rhodospirillum ferredoxins and spinach ferredoxin on the rate of $NADP^+$ reduction in washed spinach chloroplasts. Assay conditions were as in Fig. 4. The initial linear rate between 15 and 45 sec after the onset of illumination was used to calculate the rate of $NADP^+$ reduction.

TABLE III

SOME PROPERTIES OF FERREDOXINS FROM LIGHT- AND DARK-GROWN *R. rubrum* CELLS

	Light-grown cells		*Dark-grown cells*
	Type I	*Type II*	*Type II*
Non-heme iron	6	2	2
Acid-labile sulfide	6	2	2
Half-cystine residues	6	3	N.D.*
$A_{385\ nm}/A_{280\ nm}$	0.72	0.52	0.46
Molecular weight	8700	7500	N.D.*
Reduced directly by dithionite	+	−	−

* Not determined.

TABLE IV

FERREDOXIN CONTENT OF LIGHT- AND DARK-GROWN *R. rubrum* CELLS

	Bacteriochlorophyll (mg/100 g)	*Total ferredoxin (mg/100 g)*	*Relative abundance (%)*	
			Type I	*Type II*
Dark-grown cells	0.124	1.53	0	100
Light-grown cells	445.000	1.18	45	55

the ferredoxin-dependent CO_2 fixation by α-ketoglutarate synthase from *Chlorobium thiosulfatophilum*[4].

Ferredoxin from the dark-grown R. rubrum cells. The dark-grown *R. rubrum* cells yielded a ferredoxin preparation which, when chromatographed on a Sephadex G-100 column, gave only one peak. This single ferredoxin component had properties corresponding to type II of the light-grown cells: its A_{385nm}/A_{280nm} ratio was 0.46, it had 2 non-heme iron and 2 acid-labile sulfide groups per mole, and its reduction by sodium dithionite required methyl viologen (Table III and Fig. 3). It appears, therefore, that type I is the ferredoxin which is characteristic of the photosynthetic mode of life in *R. rubrum* and which is lost when this photosynthetic organism is grown heterotrophically in the dark. By contrast, type II is present in *R. rubrum* cells grown either anaerobically in the light or aerobically in the dark.

Although the light-grown cells contained both types I and II, their total ferredoxin content was lower than that of the dark-grown cells which contained only type II (Table IV). There was thus no correlation between the almost 4000-fold increase in bacteriochlorophyll content of the photosynthetically grown cells and their total ferredoxin content. The change of *R. rubrum* from a photosynthetic to a dark, heterotrophic mode of life seems to be accompanied not by a decrease in its total ferredoxin content but by a shift from two types of ferredoxin (I and II) to a single type (II).

Effect of light on ferredoxin formation in seedlings

The occurrence of ferredoxin in dark-grown *R. rubrum* cells suggested a comparison with seedlings of green plants that are capable of growing for a limited time

in the dark at the expense of food reserves stored in the seed. Table V shows that spinach seedlings germinated without exposure to light did contain appreciable amounts of ferredoxin and a small amount of chlorophyll. Upon exposure to light the ferredoxin content more than doubled, whereas the chlorophyll content increased about 500-fold. It appears, therefore, that in spinach the synthesis of ferredoxin is basically independent of light and of the synthesis of chlorophyll.

TABLE V

EFFECT OF LIGHT ON FERREDOXIN FORMATION BY SPINACH SEEDLINGS

	Ferredoxin content (mg/100 g)	*Chlorophyll content (mg/100 g)*
Seeds before germination	0.0	0.00
Seedlings germinated in the dark	1.1	0.75
Seedlings after 72 h illumination	2.7	390.00

In contrast to spinach, no clear conclusion could be drawn from the experiments with barley seedlings because their ferredoxin, when isolated, was very unstable. Ferredoxin could be demonstrated in seedlings exposed to light but not in those kept in the dark. However, because of instability of the ferredoxin, these findings could have resulted from a loss during the isolation procedure of small amounts of ferredoxin possibly present in the intact, dark seedlings.

MELANDRI *et al.*[21] reported that etiolated bean seedlings kept in the dark contained ferredoxin and that the synthesis of ferredoxin was greatly increased upon illumination. However, MELANDRI *et al.*[21] noted the possibility that, in their experiments, the ferredoxin observed in the absence of illumination might have been formed by an accidental exposure to light.

CONCLUDING REMARKS

The ferredoxin component of *R. rubrum* was found to have several distinctive features: (i) cells grown heterotrophically in the dark formed a single type (II) of ferredoxin which, unlike all other bacterial ferredoxins[3], had only 2 non-heme iron and 2 labile sulfide groups per mole; (ii) cells grown photosynthetically in the light formed in addition to type II another ferredoxin (type I) which, on the basis of its non-heme iron and labile sulfide groups (6 of each per mole) was more similar to other bacterial ferredoxins[3]; (iii) both ferredoxin components were bound, becoming solubilized after a detergent treatment.

The presence of two types of ferredoxin in a bound state in a photosynthetic bacterium is of special interest in view of the recent evidence from this laboratory[22] that spinach chloroplasts contain, in addition to the well-known soluble ferredoxin, a second, bound type of an iron-sulfur protein with an EPR spectrum characteristic of ferredoxin. The possibility arises that some type of bound ferredoxin may be a general feature of photosynthetic organisms.

ACKNOWLEDGMENT

The authors are grateful to Dr. H. Matsubara for determining the amino acid composition of the Rhodospirillum ferredoxins and wish to thank Dr. L. R. Berger for providing the *R. rubrum* culture used in their experiments.

This investigation was supported, in part, by National Science Foundation Grant GB-23796 (D.I.A.).

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