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EFFECT OF FERREDOXIN ON BACTERIAL PHOTOPHOSPHORYLATION

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SUMMARY

Recently we isolated and characterized two types of ferredoxin from photosynthetically grown *Rhodospirillum rubrum* cells. Since the ferredoxins were present in a bound form we undertook in this investigation to study the effect of ferredoxin on cyclic photophosphorylation by *R. rubrum* chromatophores that have been depleted of ferredoxin by treatment with Triton X-100. At concentrations less than 0.5 %, Triton X-100 removed over 90 % of the bound ferredoxin from the chromatophores–a depletion that was accompanied by a total loss of phosphorylating activity. The photophosphorylating activity of the depleted chromatophores was restored by the addition of ferredoxin, in the presence of a low concentration of phenazine methosulfate which without ferredoxin did not support photophosphorylation. The best reactivation of the depleted chromatophores was obtained by adding the native *R. rubrum* ferredoxins (Type I or II), *Chromatium*, or *Clostridium pasteurianum* ferredoxin. Ferredoxins from spinach or *Azotobacter vinelandii* had little effect. Photophosphorylation by reactivated chromatophores was severely inhibited by air even at high phenazine methosulfate concentrations that support high rates of photophosphorylation under air in untreated chromatophores.

INTRODUCTION

The capacity of the photosynthetic apparatus to convert radiant energy into chemical energy and to trap it in the pyrophosphate bonds of ATP, independently of the classical manifestations of photosynthesis (CO_2 assimilation and oxygen evolution), was discovered in isolated spinach chloroplasts[1]. When light-induced ATP formation was also observed in cell-free preparations of such diverse types of photosynthetic organisms as photosynthetic bacteria[2] and algae[3,4] it became evident that photophosphorylation is not peculiar to plants containing chloroplasts but is a major ATP-forming process that is independent of fermentation and respiration and occurs in all types of photosynthesis[5,6].

From the standpoint of the biochemical evolution of photosynthesis, special interest centered on cyclic photophosphorylation, the type common to chloroplasts

Abbreviation: PMS, phenazine methosulfate.

and bacterial chromatophores which yielded only ATP, required no chemical substrate, neither consumed nor produced oxygen, and was not accompanied by any measurable electron transport[1,7,6]. Cyclic photophosphorylation—so named because its operation was ascribed to a cyclic electron transport hidden in the structure of the photosynthetic apparatus[5,6]—was viewed as a primitive manifestation of photosynthetic activity, an activity that is the common denominator of plant and bacterial photosynthesis[5,6,8]. By contrast, noncyclic photophosphorylation of chloroplasts[9], in which ATP formation was stoichiometrically coupled to the evolution of oxygen and the photoreduction of an electron acceptor [first found to be $NADP^+$ (ref. 9) and later ferredoxin[10]] was clearly limited to plant photosynthesis.

Despite major similarities, a puzzling feature of cyclic photophosphorylation in freshly isolated chloroplasts, a feature which distinguished it from cyclic photophosphorylation in freshly prepared bacterial chromatophores[11,12], was a dependence on an added catalyst, for example menadione[13] or phenazine methosulfate[14], a substance that is foreign to photosynthetic cells. Moreover, unlike bacterial cyclic photophosphorylation, the one in chloroplasts was not sensitive to such characteristic inhibitors of phosphorylation as antimycin A, at low concentrations[15].

A possible explanation was that chloroplasts but not bacterial chromatophores lose a soluble constituent in the process of isolation. This explanation was consistent with a later discovery that ferredoxin, a readily soluble protein of chloroplasts, catalyzes cyclic photophosphorylation[16] and, for reasons discussed elsewhere[17], may be viewed as the physiological catalyst of that process in chloroplasts. When catalyzed by ferredoxin, cyclic photophosphorylation in chloroplasts became for the first time sensitive to inhibition by low concentrations of antimycin and oligomycin[17] and resembled in this respect cyclic photophosphorylation in bacteria[15]. Moreover, in contrast to noncyclic photophosphorylation in chloroplasts which has the short-wavelength dependence characteristic of plant photosynthesis, ferredoxin-catalyzed cyclic photophosphorylation utilizes effectively long-wavelength monochromatic light[18]—a feature reminiscent of bacterial photosynthesis.

The unified concept of photosynthesis presented earlier[8] would be materially strengthened by evidence that ferredoxin is also a catalyst of cyclic photophosphorylation in photosynthetic bacteria. Ferredoxin has long been known to be present in photosynthetic bacteria: it was isolated in 1961 in this laboratory under a different name (ref. 19 and K. TAGAWA AND M. NOZAKI, unpublished results) and as ferredoxin in 1962 (ref. 20). More recently, ferredoxin has been isolated from green and purple sulfur bacteria and shown to play an important role in photochemical NAD^+ reduction and reductive CO_2 assimilation[21–24]. There was no evidence, however, for a role of ferredoxin in bacterial photophosphorylation; even the presence of ferredoxin in phosphorylating *Rhodospirillum rubrum* chromatophores was questioned[25] and its role in *Chromatium* was ascribed not to photosynthetic CO_2 assimilation[26] but to pyruvate breakdown[27,28].

Recently we isolated and characterized two ferredoxins from *R. rubrum*, both of which were found to be present in a bound form[29,30]. In this paper we report the removal of ferredoxin from phosphorylating *R. rubrum* chromatophores and evidence which suggests that ferredoxin may be a co-factor in bacterial cyclic photophosphorylation.

METHODS

Preparation of chromatophores

Rhodospirillum rubrum S-1 cells were grown for 48 h and harvested by centrifugation as previously described[30].

The centrifuged cells were suspended in 0.05 M potassium phosphate buffer, pH 7.3 (containing 0.01 M $MgSO_4$ and 0.15 M NaCl) and sonicated for 2 min at 4° with a 20-kcycle Branson sonifier Model S-125 at an output power of 8.5 A. The sonicated cell suspension was centrifuged at 20000 × *g* for 30 min to remove broken cell debris. The precipitate was discarded and the supernatant fluid centrifuged for 1 h at 100000 × *g*. The sedimented chromatophore material was resuspended in 0.05 M Tris–HCl buffer, pH 8.0 (containing 0.01 M $MgSO_4$ and 0.15 M NaCl) and used in the experiments.

Triton treatment of the chromatophores

A 10% solution of Triton X-100 was added to the chromatophore suspension (adjusted to a bacteriochlorophyll concentration of 0.5 mg/ml with 0.05 M Tris–HCl buffer (pH 8.0) containing 0.15 M NaCl and 0.01 M $MgSO_4$) to give the desired final concentration (*cf.* Fig. 1). After standing for 1 h at 0° the mixture was centrifuged at 144000 × *g* for 60 min. The supernatant fluid was saved for the determination of ferredoxin and the sediment, resuspended in 0.05 M Tris–HCl buffer, pH 8.0 (containing 0.15 M NaCl and 0.01 M $MgSO_4$), was used in the photophosphorylation experiments.

For estimation of ferredoxin, cold acetone (—20°) was added to the supernatant solution to a final concentration of 30%. The mixture was stirred in the cold for 30 min and centrifuged at 13000 × *g* for 20 min. The supernatant fluid was passed through a 5 cm × 15 cm DEAE-cellulose column equilibrated with 0.02 M potassium phosphate buffer (pH 7.3). The column was washed with 0.02 M potassium phosphate buffer, pH 7.3 (500 ml), followed by 500 ml of 0.01 M potassium phosphate buffer (pH 7.3) containing 0.2 M NaCl. Ferredoxin was eluted from the column with 0.01 M potassium phosphate buffer (pH 7.3) containing 0.8 M NaCl, and concentrated on a short, 2 cm × 3 cm DEAE-cellulose column[30]. The concentration of ferredoxin was estimated by its substitution for spinach ferredoxin in $NADP^+$ photoreduction by washed, broken spinach chloroplasts[30].

Photophosphorylation

Photophosphorylation reactions were carried out in illuminated Warburg manometer vessels at 30° (light intensity $1.6 \cdot 10^5$ ergs/cm^2 per sec provided by Westinghouse BFA Movie Flood 375-W lamps) as described by TSUJIMOTO *et al.*[31]. The reaction was stopped after 10-min illumination by adding 0.5 ml of the reaction mixture to 1.0 ml of a solution containing 0.9 M $HClO_4$ and 0.6 M Na_2SO_4.

The ATP formed was separated from the remaining $^{32}P_i$ as described by HAGIHARA AND LARDY[32]. Aliquots containing labeled ATP, 0.5 ml, were dried in a planchet and counted in an end-window Geiger–Müller counter (Nuclear Chicago).

Other procedures

Ferredoxin was isolated from *Clostridium pasteurianum* and *Chromatium* cells using, with minor modifications, the procedure described for *R.rubrum* cells[30]. The

acetone concentration was increased from 30 to 50 % and the Sephadex column chromatography was omitted. Cytochrome c_2 and Rhodospirillum heme protein were isolated from *R. rubrum* cells according to the method of KAMEN *et al.*[33]. Bacteriochlorophyll was estimated as described by COHEN-BAZIRE *et al.*[34].

RESULTS AND DISCUSSION

Removal of ferredoxin from chromatophores

We have previously reported[30] the removal of bound ferredoxin from *R. rubrum* cells by relatively high concentrations (3–5 %) of Triton X-100. A much lower concentration, less than 0.5 %, of Triton X-100 was sufficient to solubilize over 90 % of the bound ferredoxin in isolated chromatophores. As shown in Fig. 1, the optimal Triton concentration used for a given preparation of chromatophores was proportional to its initial endogenous photophosphorylating activity. Higher concentrations of Triton impaired the subsequent restoration of photophosphorylating activity to the treated chromatophores. Lower concentrations of Triton were less effective in removing ferredoxin and resulted in a poor response to added ferredoxin.

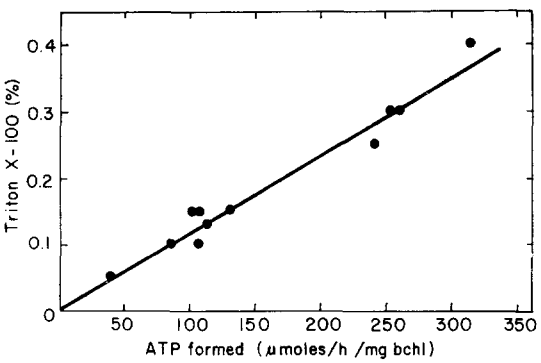


Fig. 1. Triton X-100 concentration used for extraction of ferredoxin, as a function of the initial photophosphorylating activity of *R. rubrum* chromatophores. Photophosphorylation conditions were the same as in Table I. bchl = bacteriochlorophyll.

TABLE I

EFFECT OF SOLUBILIZATION OF FERREDOXIN ON ENDOGENOUS PHOTOPHOSPHORYLATION BY *R. rubrum* CHROMATOPHORES

Triton X-100 concentration during the pretreatment was 0.25%. The photophosphorylation reaction mixture (final volume 1 ml) contained (in μmoles): Tricine buffer (pH 8.2), 100; $MgCl_2$, 2; ADP, 5; $^{32}P_i$, 5; and chromatophores containing 25 μg bacteriochlorophyll. The reaction mixture was equilibrated with argon for 10 min in the dark before illumination. Gas phase, argon.

	ATP formed (μmoles/h per mg bacteriochlorophyll)	*Ferredoxin solubilized (nmoles/g bacteriochlorophyll)*
Untreated chromatophores	240	0
Triton-treated chromatophores	0	380

The solubilization by the mild Triton treatment of the ferredoxin bound to the chromatophores was accompanied by a total loss of their endogenous photophosphorylating activity (Table I). The experiments that followed were concerned with restoration of photophosphorylating activity to ferredoxin-depleted chromatophores.

Restoration of photophosphorylation to ferredoxin-depleted chromatophores

The photophosphorylating activity of the ferredoxin-depleted chromatophores was restored, to a level higher than their initial endogenous activity, by the joint addition of ferredoxin and phenazine methosulfate (PMS) (Table II). The addition of either ferredoxin or PMS alone gave no appreciable increase in photophosphorylation.

TABLE II

RESTORATION OF PHOTOPHOSPHORYLATION BY FERREDOXIN AND PMS IN FERREDOXIN-DEPLETED CHROMATOPHORES

The reaction mixture included 20 nmoles of *Chromatium* ferredoxin and 50 nmoles of PMS. Other experimental conditions were as in Table I.

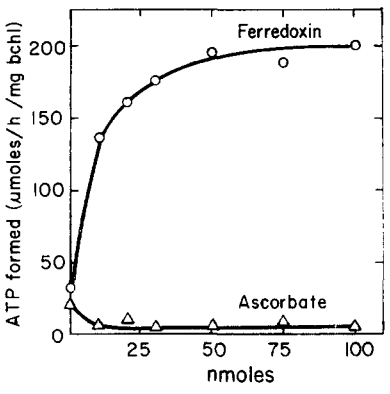
Treatment	*ATP formed (μmoles/h per mg bacteriochlorophyll)*	
	Untreated chromatophores	*Ferredoxin-depleted chromatophores*
Control	111	0
+ Ferredoxin	117	4
+ PMS	463	17
+ PMS and ferredoxin	429	175

The joint requirement of ferredoxin and PMS suggested that the restored photophosphorylating activity of treated chromatophores was of a type different from the increased photophosphorylation that results from the addition of PMS alone to untreated chromatophores[35]. Table II shows that the addition of PMS to the untreated chromatophores gave a 4-fold stimulation in photophosphorylation—a stimulation that was independent of the addition of ferredoxin. By contrast, in the ferredoxin-depleted chromatophores, the addition of PMS alone was ineffective and when PMS was added jointly with ferredoxin it acted not as a stimulant of an existing photophosphorylating activity but probably as a substitute for a missing essential cofactor of photophosphorylation that normally interacts with ferredoxin.

It is likely that the Triton treatment removed from the chromatophores not only ferredoxin but one or more other cofactor(s) of photophosphorylation for which PMS could serve as a substitute. However, attempts to replace PMS in Triton-treated chromatophores by Rhodospirillum heme protein[33], Rhodospirillum cytochrome c_2 (ref. 33), horse heart cytochrome *c*, ubiquinone-10, dichlorophenol indophenol, or ascorbate (up to 1 mM) were, in the main, unsuccessful.

Several investigators have observed that in washed bacterial chromatophores cyclic photophosphorylation is activated by catalytic amounts of reductants[35–37]. A systematic investigation of this effect in washed *R. rubrum* chromatophores showed that ascorbate was a most effective reductant in restoring photophosphorylating

activity[38]. The question arose whether the restorative effect of added ferredoxin (in the presence of PMS) on photophosphorylation by ferredoxin-depleted chromatophores could also be achieved by adding ascorbate instead of ferredoxin. Fig. 2 shows that ascorbate was wholly ineffective as a substitute for ferredoxin.



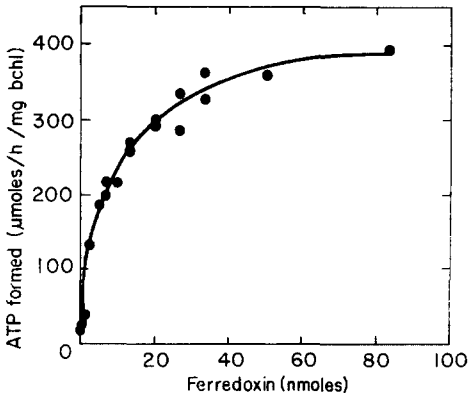


Fig. 2. Ineffectiveness of ascorbate as a substitute for ferredoxin in reactivating photophosphorylation in ferredoxin-depleted chromatophores. 50 nmoles PMS was added throughout. Other experimental conditions were as in Table I.

Fig. 3. Effect of ferredoxin concentration on the restoration of photophosphorylation in ferredoxin-depleted chromatophores. Where shown, duplicate values were from two different experiments. The ferredoxin used was isolated from *C. pasteurianum*. 50 nmoles of PMS was added throughout. Other experimental conditions were as in Table I.

The effect of concentration of added ferredoxin on photophosphorylation by ferredoxin-depleted chromatophores is shown in Fig. 3. Photophosphorylation was greatly increased by low concentrations of ferredoxin: the increase was linear up to about 3 μM ferredoxin and leveled off at about 30 μM ferredoxin.

Table III shows the effect of different ferredoxins on the restoration of photophosphorylation in ferredoxin-depleted chromatophores. Ferredoxins from *R. rubrum*,

TABLE III

EFFECT OF DIFFERENT FERREDOXINS ON RESTORATION OF PHOTOPHOSPHORYLATION IN FERREDOXIN-DEPLETED CHROMATOPHORES

50 nmoles of PMS was added in all cases and 20 nmoles each of the respective ferredoxins. Other experimental conditions were as described in Table I.

Ferredoxin added	*ATP formed (μmoles/h per mg bacteriochlorophyll)*
None	53
Rhodospirillum rubrum, type I	256
Rhodospirillum rubrum, type II	225
Chromatium D	241
Clostridium pasteurianum	257
Spinach	95
Bacillus polymyxa	84
Azotobacter vinelandii	57

Chromatium, and *C. pasteurianum* but not those from spinach, *Bacillus polymyxa* and *Azotobacter vinelandii* were capable of restoring photophosphorylation. No correlation was found between the restorative ability of a ferredoxin and its non-heme iron and acid-labile sulfide content. Thus, spinach ferredoxin with two non-heme iron and labile sulfide groups had a small effect but *R. rubrum* ferredoxin, Type II, with the same iron and sulfide content[30] was very effective and comparable to *R. rubrum* ferredoxin Type I with six (ref. 30) and *Chromatium* and *Clostridium* ferredoxins, each with eight iron and sulfide groups[24]. In general, the ferredoxins from photosynthetic bacteria and the obligate anaerobe *C. pasteurianum* were very effective in restoring photophosphorylation to ferredoxin-depleted *R. rubrum* chromatophores, whereas ferredoxins from plants or aerobic bacteria were distinctly less so.

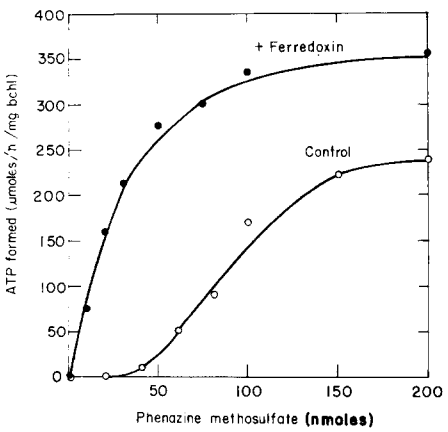


Fig. 4. Effect of phenazine methosulfate concentration with or without added ferredoxin on photophosphorylation by ferredoxin-depleted chromatophores. 20 nmoles of *C. pasteurianum* ferredoxin was added where indicated. Other experimental conditions were the same as in Table I.

The effect of PMS concentration on the reactivation of photophosphorylation in ferredoxin-depleted chromatophores, with or without added ferredoxin, is shown in Fig. 4. In the presence of added ferredoxin, low concentrations of PMS strongly reactivated photophosphorylation but were ineffective in the absence of ferredoxin. In the presence of ferredoxin, the effect of PMS concentration was linear up to 50 μM and reached saturation at about 100 μM PMS. It appears likely that only at low concentrations of PMS and added ferredoxin is a cyclic electron transport pathway reestablished which resembles the physiological one.

Without added ferredoxin, additions of PMS gave a sigmoid curve. PMS began reactivating photophosphorylation in ferredoxin-depleted chromatophores only at concentrations greater than 50 μM; saturation was reached at about 200 μM (Fig. 4, lower curve) but even at this (or higher) level of PMS the restored photophosphorylation never attained the rate observed in the presence of ferredoxin. The occurrence of photophosphorylation in Triton-treated *R. rubrum* chromatophores (without added ferredoxin) at very high concentrations of PMS (400 μM) was previously reported by HORIO AND YAMASHITA[39].

Effect of antimycin A

As already mentioned, a characteristic feature of endogenous bacterial photophosphorylation is that it is inhibited by antimycin A. This inhibition, and a similar one by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide is reversed in the presence of PMS (refs. 35, 40)—an observation that is explained by PMS serving as a by-pass for an electron transfer step(s) between cytochromes *b* and *c* blocked by these inhibitors[41]. It was of interest, therefore, to determine the sensitivity to antimycin A of the restored photophosphorylation.

TABLE IV

EFFECT OF ANTIMYCIN A ON PHOTOPHOSPHORYLATION BY UNTREATED AND FERREDOXIN-DEPLETED CHROMATOPHORES

Final concentrations: antimycin A, $1.8 \cdot 10^{-7}$ M; PMS, $5 \cdot 10^{-5}$ M; *Chromatium* ferredoxin, $2.0 \cdot 10^{-5}$ M. Other conditions were as in Table I.

	ATP formed (μmoles/h per mg bacteriochlorophyll)		
	Untreated chromatophores		*Ferredoxin-depleted chromatophores*
	Endogenous	*+ PMS*	*PMS + ferredoxin*
Control	109	230	156
+ Antimycin A	0	218	164

Table IV shows that the restored photophosphorylation in the ferredoxin-depleted chromatophores was not inhibited by antimycin A. The endogenous photophosphorylation of the untreated chromatophores was, as expected, completely inhibited by antimycin A, an inhibition that disappeared (with a doubling of activity) in the presence of PMS. It appears, therefore, that the antimycin-sensitive site between cytochromes *b* and *c* was by-passed in the reactivated system. Antimycin A would not be expected to affect the electron step(s) catalyzed by ferredoxin.

Effect of air

Endogenous cyclic photophosphorylation by *R. rubrum* chromatophores depends on a proper intra-chromatophore oxidation–reduction balance that is readily influenced by the gaseous environment[38,42]. It proceeds optimally under anaerobic conditions and is severely inhibited by air[38,39,42]. By contrast, air does not inhibit bacterial photophosphorylation in the presence of high concentrations of PMS. Under such conditions, the same maximal rate of photophosphorylation by *R. rubrum* chromatophores was observed whether air was admitted or excluded[39]. Thus sensitivity to air may be used to distinguish the endogenous type of cyclic photophosphorylation in *R. rubrum* chromatophores from that catalyzed by high concentrations of PMS.

The effect of air on photophosphorylation by the ferredoxin-depleted and untreated chromatophores as a function of PMS concentration is shown in Fig. 5. In untreated chromatophores, endogenous photophosphorylation (at low PMS) was severely inhibited by air. The inhibition by air was, as expected, reversed by the addition of PMS which, up to a concentration of about 0.2 mM, gave a linear increase

in photophosphorylation that reached a level about equal to that under argon (Fig. 5, left). Higher concentrations of PMS produced no appreciable increase in the rate of photophosphorylation.

A different relation between PMS and photophosphorylation under aerobic conditions was observed in the ferredoxin-depleted chromatophores, supplemented by added ferredoxin. In this reconstituted system, air severely inhibited photophosphorylation at all concentrations of PMS and the rate of the process under aerobic conditions was always drastically lower than that under anaerobic conditions (Fig. 5, right). Under air, PMS concentrations lower than 0.03 mM supported no measurable photophosphorylation. Photophosphorylation slowly increased at higher PMS concentrations and reached saturation at 0.2 mM PMS. No further increase in the rate of photophosphorylation under air was observed, even when the concentration of PMS was increased to 0.5 mM.

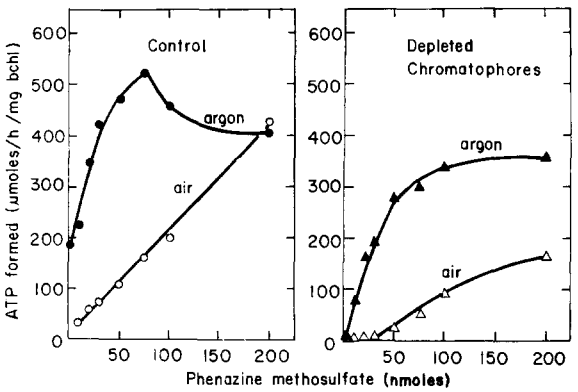


Fig. 5. Effect of air on photophosphorylation in untreated and ferredoxin-depleted chromatophores. 20 nmoles of *C. pasteurianum* ferredoxin was added in all cases except in the Control, air treatment. Other experimental conditions were as in Table I except that in the air treatments pre-equilibration with argon was omitted.

In the reactivated system under air, the plot of photophosphorylation rate versus PMS concentration gave a sigmoidal curve (Fig. 5, lower right) which was similar to the sigmoid curve for anaerobic photophosphorylation by ferredoxin-depleted chromatophores that were not supplemented with ferredoxin (Fig. 4). This similarity suggests that under aerobic conditions photophosphorylation by ferredoxin-depleted chromatophores may have also been impeded by an insufficiency of ferredoxin, even when soluble ferredoxin was added to the reaction mixture. It is possible that the added ferredoxin, after being photochemically reduced[24], was being reoxidized by air and thereby acted as an electron leak for the light-induced cyclic electron flow.

According to this interpretation, ferredoxin serves as an endogenous cofactor of bacterial cyclic photophosphorylation and, when photoreduced by bacteriochlorophyll, is reoxidized by the cyclic electron transport chain—a reoxidation that may be artificially accelerated by added PMS. Cyclic photophosphorylation is impaired by a deficiency of ferredoxin brought about either by its extraction from chromatophores or by oxidation by air of ferredoxin added to ferredoxin-depleted chromatophores.

CONCLUDING REMARKS

Recognition of the key role of ferredoxin in photosynthesis[43,17] has recently been followed by evidence that ferredoxin is present in the photosynthetic apparatus in more than one form. Two membrane-bound types of ferredoxin, both found to be effective in restoring photophosphorylation to ferredoxin-depleted chromatophores (Table III) have recently been isolated from *R. rubrum*[30]. Other recent work in this laboratory has yielded evidence for an iron-sulfur protein (with an EPR spectrum characteristic of ferredoxin) that is bound to the thylakoids of spinach chloroplasts[44] and appears to be distinct from the soluble form of chloroplast ferredoxin that has been intensively investigated in the past[43,17]. The bound chloroplast ferredoxin is photoreduced at 77°K and may therefore be considered to be a primary electron acceptor from excited chlorophyll[44]. The ability of bacterial chromatophores to photoreduce ferredoxin has been demonstrated for *Chlorobium thiosulfatophilum*[23,45] but it has not yet been determined whether such photoreduction would also occur at liquid nitrogen temperature in this and in other photosynthetic bacteria.

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