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THE NITROGEN FIXATION SYSTEM OF PHOTOSYNTHETIC BACTERIA

II. *CHROMATIUM* NITROGENASE ACTIVITY LINKED TO PHOTOCHEMICALLY GENERATED ASSIMILATORY POWER

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SUMMARY

The nitrogenase activity (measured by N_2 or acetylene reduction) of cell-free extracts of the photosynthetic bacterium *Chromatium* was coupled to photochemically generated ATP and reductant. The ATP was formed through cyclic photophosphorylation by bacterial chromatophores. The reductant (reduced ferredoxin) was generated by a heated preparation (incapable of O_2 and ATP production) of spinach chloroplasts. The nitrogenase activity of *Chromatium* extracts was supported by reduced *Chromatium* or *Clostridium pasteurianum* ferredoxin but not by that of spinach chloroplasts.

INTRODUCTION

It is now well established that the role of light in photosynthetic CO_2 assimilation by chloroplasts is to generate assimilatory power, consisting of ATP and of reduced ferredoxin which in turn generates reduced NADP (see review[1]). Earlier work on nitrogen fixation by *Chromatium* extracts led to the conclusion that "the role of light in the photofixation of nitrogen by *Chromatium* is to generate electrons with a reducing potential equal to that of hydrogen[2]". This conclusion was reinforced by the later recognition that the *Chromatium* protein called "pyridine nucleotide reductase[3]" is a ferredoxin with a reducing power about equal to that of H_2 (refs. 4, 5) and the findings that reduced ferredoxin is the electron donor for the nitrogenase system of nonphotosynthetic bacteria[6,7].

Since nitrogen fixation was also found to require ATP[8,9] the energy requirements for this process (ATP and reduced ferredoxin) are now known to be qualitatively the same as those for CO_2 assimilation. Since photosynthetic bacteria like *Chromatium* are obligate phototrophs, they would be expected to have evolved a link between light reactions and nitrogen fixation similar to that between light reactions and CO_2 assimilation.

This communication reports nitrogen fixation and acetylene reduction by a cell-free extract of *Chromatium* supported by light-driven reactions. It was possible to supply ATP through photophosphorylation by bacterial chromatophores and to

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

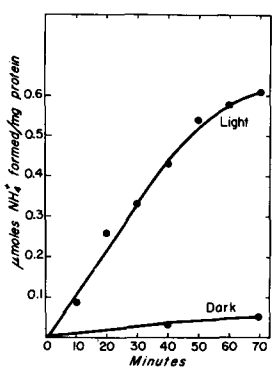
generate reduced ferredoxin photochemically by illuminated spinach chloroplast fragments.

METHODS

Chromatium Strain D was grown, washed and harvested as described in the companion paper[10]. Washed cells were resuspended in 0.01 M HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2) and broken in a Beckman Ribi cell fractionator. Whole cells and debris were removed by centrifugation at 33000 × *g* for 20 min. The resulting supernatant fluid was then centrifuged at 144000 × *g* for 90 min. All centrifugations were carried out in capped tubes gassed with argon. The pellet was resuspended in 0.01 M HEPES buffer (pH 7.8) and used as the chromatophore preparation. Chromatophores from *Rhodospirillum rubrum* were prepared in the same way from sonicated cells and retained phosphorylating activity for at least 3 days at 3°. The ferredoxins used in this study were isolated and purified as described previously[4,5]. The ferredoxin concentrations shown in Fig. 3 were calculated from the recently published molar extinction coefficients[11]. The spinach chloroplast fragments were prepared by the method of WHATLEY AND ARNON[12] and were heated for 5 min at 55° to destroy their O_2-evolving and phosphorylating activity. Chlorophyll was determined as described by ARNON[13]. Photophosphorylation was assayed colorimetrically by the disappearance of inorganic phosphate[14], and ammonia and ethylene as described in the companion paper[10].

RESULTS AND DISCUSSION

The nitrogenase activity in the cell-free extract of *Chromatium* was followed by measuring the production of ammonia from N_2 or ethylene from acetylene. Table I shows the requirements for N_2 fixation and acetylene reduction when photoreduced ferredoxin is the reductant. Nitrogen and acetylene reduction occurred only in the light and was dependent on ferredoxin, an electron donor system (ascorbate–dichloro-



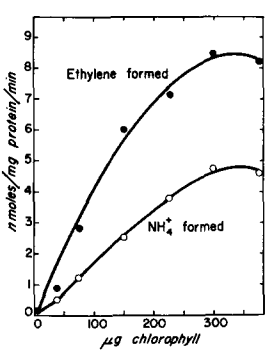


Fig. 1. Time course of light-dependent N_2 fixation by a cell-free extract of *Chromatium*. Experimental conditions were as given for Table I, except that 5 mg 144000 × *g* supernatant extract were used.

Fig. 2. Effect of chlorophyll concentration on N_2 fixation and acetylene reduction by a cell-free extract of *Chromatium*. Experimental conditions were as described for Table I, except that 7.8 mg 144000 × *g* supernatant extract were used.

TABLE I

REQUIREMENTS FOR LIGHT-DEPENDENT N_2 FIXATION AND ACETYLENE REDUCTION BY A CELL-FREE EXTRACT OF *Chromatium*

The complete system contained in a total volume of 1.5 ml: heated spinach chloroplast fragments (equivalent to 185 μg chlorophyll); 100 μmoles HEPES buffer (pH 7.2), 10 μmoles ascorbate, 0.05 μmole DCIP, 10 μmoles $MgCl_2$, 5 μmoles ATP, 50 μmoles creatine phosphate, 0.2 mg creatine phosphokinase, 200 μg ferredoxin from *C. pasteurianum* and 6.6 mg *Chromatium* extract. Gas phase was N_2 or 0.1 atm acetylene and 0.9 atm argon, depending on type of nitrogenase assay used. Temp., 30°, light intensity, 20 000 lux.

Treatment	*nmoles formed per mg protein per min*	
	Ammonia	*Ethylene*
Complete	4.8	8.2
Complete, dark	0	0
Minus ferredoxin	0.1	<0.01
Minus chloroplasts	0.5	<0.01
Minus (creatine phosphate *plus* ATP)	0.2	0
Minus (ascorbate *plus* DCIP)	0	<0.01
Minus Mg^{2+}	2.0	—
Complete but N_2 or acetylene replaced by argon	0	0

phenolindophenol (DCIP)) for the chloroplasts, chloroplast fragments and an ATP-generating system.

Time course data (Fig. 1) showed that the reaction was linear for about 40 min, leveling off in 80 min. On the basis of these results, a 30-min reaction period was used for routine assays.

The amount of acetylene or N_2 reduced in the light varied with the concentration of added chlorophyll in a manner shown in Fig. 2. The data shown in Fig. 2 and in Table I gave molar ratios of ethylene/ammonia of 1.7 and 1.8, respectively—slightly higher than the theoretical value of 1.5. These values are in close agreement with those for other N_2-fixing systems[15].

The effect of ferredoxins from three different sources, *C. pasteurianum*, *Chromatium* and spinach, on N_2 fixation by *Chromatium* extracts is shown in Fig. 3. Spinach

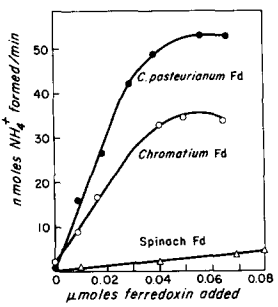


Fig. 3. Dependence of N_2 fixation on the concentration of photochemically reduced ferredoxins. The ferredoxins used in this experiment were from three sources: *C. pasteurianum*, *Chromatium* and spinach. Except for varying ferredoxin concentrations as indicated and using 7.8 mg 144 000 × *g* supernatant extract, experimental conditions were as described for Table I. Fd, ferredoxin.

ferredoxin was only about 8–10 % as active as the bacterial ferredoxins. This result is not unusual since spinach ferredoxin has been shown to be relatively ineffective in mediating several reactions catalyzed by clostridial enzymes[16]. Fig. 3 also shows that *C. pasteurianum* ferredoxin was more active in N_2 fixation than *Chromatium* ferredoxin. Other experiments have shown that ferredoxin from *Chlorobium thiosulfatophilum* is as active as that of *C. pasteurianum* in the nitrogenase reaction.

TABLE II

NITROGENASE ACTIVITY SUPPORTED BY LIGHT ENERGY

The complete system contained in a total volume of 1.5 ml: 100 μmoles HEPES buffer (pH 7.8), 5 μmoles $MgCl_2$, 10 μmoles ADP, 10 μmoles KH_2PO_4, 6 mg *Chromatium* extract and *Chromatium* or *R. rubrum* chromatophores as indicated. Gas phase, 0.1 atm acetylene and 0.9 atm argon. After 30 min pre-incubation of the above components in the light (20000 lux), 10 μmoles ascorbate, 0.05 μmole DCIP, 200 μg ferredoxin and heated spinach chloroplast fragments (300 μg chlorophyll) were added anaerobically. The complete reaction was then incubated 20 min in the light (20000 lux) at 30°.

ATP source	*μmoles ATP generated per 30 min pre-incubation*	*nmoles ethylene formed per min*
None	—	0.6
ATP-generating system (creatine phosphate–creatine phosphokinase	>50	65
Light		
Chromatium chromatophores (1.0 mg bacteriochlorophyll)	9.2	20
R. rubrum chromatophores (0.1 mg bacteriochlorophyll)	6.0	15
Dark		
Chromatium chromatophores (1.0 mg bacteriochlorophyll)	0	0

Table II shows that photophosphorylation by *Chromatium* or *R. rubrum* chromatophores can supply the ATP requirement for nitrogen fixation. ATP was formed during a preillumination period of the chromatophores prior to the addition of chloroplasts—a procedure found necessary, probably to insure that the phosphorylating system was properly poised[17]. The data in Table II indicate that all the energy requirements for nitrogen fixation (ATP and reductant) can be provided by light-driven reactions.

Photoreduction of ferredoxin by bacterial chromatophores has been achieved in Chlorobium[18,19] and similar attempts with *Chromatium* chromatophores are continuing. In the meantime, the use of illuminated spinach chloroplasts as a source of reducing power has already established that photochemically reduced *Chromatium* ferredoxin can transfer electrons directly to the *Chromatium* nitrogenase system.

Unlike such artificial reductants as dithionite, the reducing power generated photochemically by chloroplasts cannot be coupled to the nitrogenase system directly but only through intermediate electron carriers. Thus, the use of illuminated chloroplasts as a source of reducing power may provide a useful tool for the identification of nitrogenase-linked cellular electron carriers even in nonphotosynthetic, N_2-fixing organisms.

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