BBA 45600

OXYGEN INHIBITION OF NITROGEN FIXATION IN CELL-FREE PREPARATIONS OF BLUE-GREEN ALGAE

P. FAY AND ROSALIE M. COX

Department of Botany, Westfield College, University of London, London, N.W. 3, and Department of Botany, The University, Bristol (Great Britain)

(Received May 12th, 1967)

SUMMARY

1. Factors inhibiting N_2 fixation in cell-free preparations of two blue-green algae, *Anabaena cylindrica* and *Chlorogloea fritschii* were investigated.

2. ^{15}N uptake by particulate cell-free fractions of *A. cylindrica* was less in the light than in the dark, indicating some photo-inhibition of N_2 fixation.

3. Anaerobic conditions during preparation and incubation of particulate cell-free fractions of *C. fritschii* greatly increased their N_2-fixing ability.

4. Soluble cell-free material was found to have an inhibitory effect on N_2 fixation by particulate cell-free preparations obtained from *A. cylindrica*. This inhibition was approximately proportional to the amount of soluble fraction supplied to the particulate material. Dialysis of the soluble cell-free fraction did not remove the inhibition which has therefore been attributed to the presence of a soluble enzyme, possibly an oxidase.

5. Dialysis of the soluble cell-free fraction or of particulate material suspended with soluble cell-free fraction resulted in a slight decrease of ^{15}N uptake, indicating the removal by dialysis of small-molecule factors required for N_2 fixation.

6. The presence of sulphydryl agents increased the rate of N_2 fixation in cell-free preparations of *A. cylindrica*.

INTRODUCTION

N_2-fixing activity in two blue-green algae, *Anabaena cylindrica* and *Chlorogloea fritschii* appears to be located on the pigmented lamellar system of the cell[1,2]. Cell-free suspensions of fragmented lamellae obtained from these organisms fix N_2 even when largely washed free from soluble cell components[1,2]. Since the addition of pyruvate to particulate preparations of *A. cylindrica* has been found to stimulate N_2 fixation (R. M. COX AND P. FAY, unpublished observation), it was of interest to find out whether endogenous soluble components are required for N_2 fixation.

Cell-free extracts of blue-green algae have hitherto been prepared and frequently incubated aerobically. It was thought desirable to investigate whether O_2 has any effect on the N_2-fixing activity of these preparations. Extracts of the anaerobic bacterium *Clostridium pasteurianum* are routinely prepared anaerobically since O_2 inhibites N_2 fixation both in intact cells and cell-free extracts[3,4]. The aerobic *Azoto-*

bacter vinelandii requires O_2 for growth and N_2 fixation[5] and cell-free N_2 fixation has usually been studied with material prepared aerobically from this bacterium[5–8]. However, BULEN[9] has recently reported that anaerobic preparation increases N_2-fixing activity in cell-free extracts of *A. vinelandii*.

Results communicated in this paper indicate that the N_2-fixing system of blue-green algae is susceptible to oxidation. The soluble material of the cell contains some large-molecule compound inhibitory to N_2 fixation and also some small-molecule factors required for N_2 fixation.

METHODS

A. cylindrica and *C. fritschii* were grown as described previously[10,11]. For all experiments cell material was transferred into 50 ml fresh medium and N-starved, as this procedure increased ^{15}N uptake during subsequent incubation in intact cells[10,12]. The N-starved material was concentrated in 20 ml medium and disrupted either at 2100 atm in a French press[13] precooled to 4° or by 5 min sonic oscillation in a 60-W

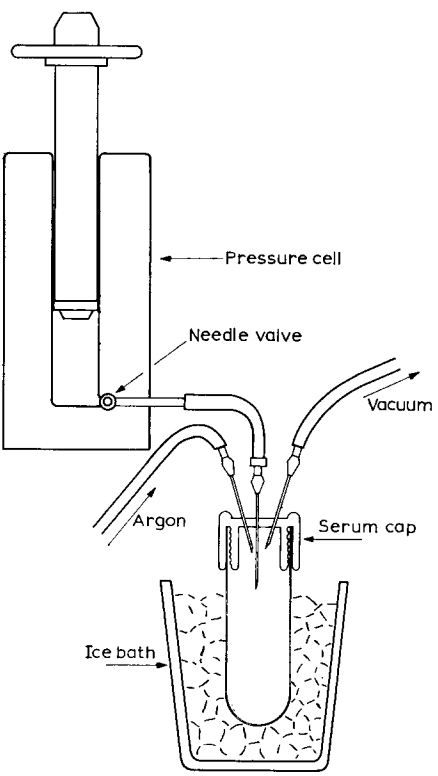

Fig. 1. Anaerobic preparation of cell-free extracts. A centrifuge tube sealed with a serum rubber cup was immersed in an ice bath and attached to the outlet of the French press[13] by means of silicone tubing and a syringe needle piercing the rubber cap. Two further syringe needles connected the centrifuge tube to an argon gas line and a vacuum line, respectively. These allowed a continuous stream of argon to flush both the collecting tube and the outlet tube, up to the needle valve of the French press. Thus the smashed cells were instantly under anaerobic conditions. Centrifugation of the extract and transfers of material were carried out under argon.

M.S.E. sonic disintegrator. The crude cell homogenate prepared in the French press was centrifuged at 2000 × *g* for 10 min, while that obtained by sonication was centrifuged at 10000 × *g* for 10 min, in order to remove intact cells and cell-wall debris. The supernatant was then used to obtain pellet fractions precipitating between either 2000–35000 × *g* or between 10000–100000 × *g* by centrifugation at the higher speed for 1 h. The prepared particulate fractions were homogenised in a glass homogeniser and washed in fresh sterile medium before final resuspension in a similar medium. All operations were carried out at 4°. A method for preparing extracts anaerobically is described with Fig. 1.

Samples of resuspended particulate material were incubated in Warburg flasks filled with the required gas or gas mixture, including ^{15}N-labelled N_2. Following incubation, ^{15}N uptake was estimated by the method of BURRIS AND WILSON[14] using a MS 3 Associated Electrical Industries Ltd. mass spectrometer. Total N determinations were made using a standard Nessler's procedure after digestion and distillation[14]. NH_3 accumulation in the extracts was measured by the micro-diffusion method of DILWORTH *et al.*[15].

RESULTS

Experiments with intact cells of *A. cylindrica* and *C. fritschii* have shown N_2 fixation to be closely dependent upon a supply of carbon skeletons normally provided by photosynthesis (but see FAY[12]) to assimilate the ammonia produced[10,11,16]. Suggestions have been made that light may promote N_2 fixation in ways other than the production of carbon skeletons[17,18], *i.e.* by supplying reducing potential and/or ATP. Our primary aim was to see whether some stimulation of cell-free N_2 fixation unrelated to CO_2 assimilation could be established when particulate cell-free material was incubated in the light.

Table I demonstrates that rather than any stimulation of N_2 fixation in light occurring, light incubation decreased N_2 uptake as compared to N_2 uptake in dark-incubated samples. This has been usually but not invariably observed[2]. WHATLEY[19] has shown that in isolated chloroplasts light stimulates the oxidation of certain intermediates of cyclic photophosphorylation. It was therefore considered from the results of Table I that photo-oxidation of some factors required in a reduced state for N_2 fixation may have occurred.

TABLE I

N_2 FIXATION IN A 2000–35000 × *g* PARTICULATE CELL-FREE FRACTION OF *A. cylindrica* IN THE LIGHT AND IN THE DARK

4-ml aliquots of the suspension (average N content = 50 μg/ml) were incubated in the light (600 foot-candles) or in the dark for 1.5 h at 30°. Gas phase: 10% N_2 (60% ^{15}N), 10% O_2 and 80% argon. Results are averages of duplicate determinations.

Treatment	*Atom per cent ^{15}N excess*		
	Expt. 1	*Expt. 2*	*Expt. 3*
Light	0.030	0.024	0.009
Dark	0.047	0.050	0.014

To test the possibility that the N_2-fixing system is susceptible to oxidation, particulate preparations from *C. fritschii* were obtained aerobically and anaerobically (Fig. 1), and both exposed to $^{15}N_2$ with or without O_2 in the dark. The results set out in Table II clearly show that anaerobic treatment increased the rate of ^{15}N-fixation. Anaerobic incubation appeared to be more important than anaerobic preparation in preserving N_2-fixing activity. This is probably because preparation of extracts was carried out at 4° where chemical reactions would be slow, while oxidation would be more rapid during the incubation carried out at 35°. In view of this sensitivity of the N_2-fixing system of blue-green algae to oxidation, it is probable that the inhibitory effect of light on N_2 fixation is one of photo-oxidation.

More information about inhibition of the N_2-fixing system by oxidation came from an experiment which was designed to determine whether the soluble cell-free fraction, usually rejected, might contain some factors required for N_2 reduction by particulate material from *A. cylindrica*. The results of this experiment, presented in Table III, clearly show that instead of effecting any enhancement, addition of the supernatant fraction invariably depressed the rate of N_2 fixation by the particulate material. This inhibition was approximately proportional to the amount of supernatant fluid added to the particulate material. It was thought that this rather un-

TABLE II

EFFECT OF O_2 ON N_2 FIXATION BY A 2000–35000 × *g* PARTICULATE CELL-FREE FRACTION FROM *C. fritschii*

4-ml aliquots of the suspension (170 μg N/ml) were incubated in the dark at 35° for 1 h. Gas phase: 10% N_2 (45.5% ^{15}N), *plus* or *minus* 10% O_2 and the rest argon. The anaerobic flasks contained 0.5 ml $CrCl_2$ solution (prepared according to MYERS[20]) in the side arm to absorb any traces of O_2. Values are the averages of duplicate determinations.

Preparation	*Incubation*	*Atom per cent ^{15}N excess*
Aerobic	Aerobic	0.017
Anaerobic	Aerobic	0.022
Aerobic	Anaerobic	0.042
Anaerobic	Anaerobic	0.087

TABLE III

EFFECT OF SOLUBLE CELL-FREE MATERIAL UPON N_2 FIXATION BY A 2000–35000 × *g* PARTICULATE FRACTION FROM *A. cylindrica*

Equal quantities of particulate material were suspended in either 100% fresh sterile medium, medium containing 10% 35000 × *g* supernatant fluid or with medium containing 50% 35000 × *g* supernatant. 4-ml samples were incubated in the light (600 foot-candles) at 30° for 1 h. Gas phase: 15% N_2 (53.5% ^{15}N), 10% O_2 and 75% argon. Results are averages of duplicate determinations.

Treatment	*Expt. 1*			*Expt. 2*		
	Total N (μg/ml)	*N fixed (μg)*	*Inhibition (%)*	*Total N (μg/ml)*	*N fixed (μg)*	*Inhibition (%)*
No supernatant	19	0.014	—	20	0.023	—
10% supernatant	30	0.011	19	25	0.017	26
50% supernatant	42	0.006	55	37	0.011	52

expected effect might be attributed to the presence of oxidase enzymes in the soluble fraction. Under aerobic conditions, these enzymes could bring about the oxidation of some component required for N_2 reduction. Presumably in intact cells, cellular organisation is such that the sites of N_2 fixation are maintained under reducing conditions. When cell organisation is broken down, O_2 may come in direct contact with the sites of N_2 fixation, and can be taken up in the presence of suitable oxidases. Analogy may be drawn to the oxidase enzymes present in storage tissues of higher plants which catalyze oxidation of phenolic compounds when cells are damaged and their contents become exposed to air[21].

In another experiment, soluble cell-free material was first dialysed against fresh medium before examining its effect on N_2 fixation by the particulate fraction. Details of this experiment are given in Table IV. Dialysis of either supernatant fluid alone or particulate matter suspended in supernatant fluid resulted in no lessening but in a slight increase of the inhibitory effect, as compared with the effect of undialysed supernatant, upon N_2 fixation by the particulate fraction. It was therefore concluded that the inhibitory agent present in the soluble fraction must be a fairly large-molecule compound, possibly an enzyme. The additional slight decrease in the rate of N_2 fixation which occurred when the particulate matter was previously dialysed with supernatant fluid (Table IV, Expt. 1) or supplied with dialysed soluble cell-free fraction (Table IV, Expt. 2), apparently indicates that some small molecules, required for N_2 reduction, were removed by dialysis.

Compounds containing sulphydryl groups prevent the oxidation of phenolic substrates in damaged storage tissues[21]. The presence of mercaptoethanol also considerably increased the rate of ^{15}N uptake by particulate fractions of *A. cylindrica* under aerobic conditions (Table V). The effect of sulphydryl compounds on cell-free

TABLE IV

EFFECT OF DIALYSIS ON N_2 FIXATION BY 2000–35000 × *g* PARTICULATE CELL-FREE FRACTIONS OF *A. cylindrica*

Expt. 1: particulate material was suspended in medium containing 10% supernatant fluid, one part of this suspension was dialysed in Visking tubing at 4° in the dark for 24 h against four 500-ml changes of fresh sterile medium, while the other part was kept under the same conditions but not dialysed. Expt. 2: one part of the supernatant fraction used in this experiment was dialysed as before, the remainder being retained under the same conditions but not dialysed. Both solutions were used to resuspend freshly prepared particulate material. Particle-density of suspensions was approximately the same. All samples were incubated in the light (600 foot-candles) at 30° for 1 h. Gas phase: 10% N_2 (54% ^{15}N), 10% O_2 and 80% argon. Values are averages of duplicate determinations. Results of the two experiments are not directly comparable because of different particle-density of suspensions.

Treatment	*Total N* (*μg/ml*)	*N fixed* (*μg*)
Expt. 1:		
Particulate fraction suspended in 10% soluble fraction and dialysed	70	0.016
Particulate fraction suspended in 10% soluble fraction and not dialysed	70	0.024
Expt. 2:		
Particulate fraction suspended without added soluble fraction	50	0.141
Particulate fraction suspended in 10% soluble fraction	57	0.051
Particulate fraction suspended in 10% dialysed soluble fraction	66	0.046

N_2 fixation was also tested under anaerobic conditions in the dark (Table VI). N_2 fixation in this experiment was measured by means of NH_3 accumulation[15]. This could not be detected in the absence of sulphydryl agents but mercaptoethanol, cysteine and sodium thioglycollate were all effective in stimulating NH_3 accumulation. Since these agents could promote N_2 fixation in the absence of O_2 in the dark, it is questionable whether their action was principally one of protecting the N_2-fixing system from oxidation. DUA AND BURRIS[22] found that sulphydryl compounds could partially restore activity to the cold-inactivated N_2-fixing complex of *C. pasteurianum*. The action of these agents is thought not to be principally one of maintaining reducing conditions but probably to cause the reaggregation of the partially denatured N_2-fixing system into an effective unit. It is possible that the effect of sulphydryl agents on the N_2-fixing system of *A. cylindrica* is 2-fold, *i.e.* the protection of reducing agents from oxidation and the reconstitution of the partially denatured enzyme system.

TABLE V

EFFECT OF MERCAPTOETHANOL ON N_2 FIXATION BY A 2000–35000 × *g* PARTICULATE FRACTION OF *A. cylindrica*

4-ml aliquots of the suspension were prepared with or without 0.002 M mercaptoethanol and incubated for 1 h at 30° in the light (600 foot-candles). Gas phase: 9% N_2 (55% ^{15}N), 8% O_2 and 83% argon. Values are averages of duplicate determinations.

Treatment	*Total N* (*μg/ml*)	*μg N fixed per mg total N*
No mercaptoethanol	50	0.020
Mercaptoethanol added	30	0.436

TABLE VI

EFFECT OF SULPHYDRYL COMPOUNDS ON NH_3 ACCUMULATION IN A 10000–100000 × *g* PARTICULATE FRACTION OF *A. cylindrica*

3-ml aliquots of suspension were incubated in the light (600 foot-candles) for 1.5 h at 30°. Gas phase: 100% N_2. Additional compounds were supplied at a final concn. of 0.002 M. Incubation was terminated by adding 1 ml satd. K_2CO_3 solution to the samples, and the NH_3 evolved was absorbed in a drop of 0.5 M H_2SO_4 hanging on an etched glass rod which was fixed in the stopper of each reaction flask. Flasks were shaken overnight at 30° and the glass rods then washed into Nessler's reagent. Values, obtained after subtraction of the initial NH_3 content in the samples, are averages of triplicate determinations.

Treatment	*Total N* (*μg/ml*)	*μg NH_3 accumulated per mg total N*
No additions	134	−0.94
plus mercaptoethanol	125	+0.64
plus cysteine	108	+2.71
plus sodium thioglycollate	115	+4.35

DISCUSSION

Evidence presented above suggests that the N_2-fixing system as prepared hitherto from blue-green algae is inefficient. It is probable that the preparation

procedure damages the organisation of the N_2-fixing apparatus and alters the reducing conditions originally present at the sites of N_2 fixation. Though inhibition by soluble cell components is appreciably reduced by washing the active particulate material, some of the soluble cell contents appear to be trapped by the lamellar vesicles formed during cell disruption (R. M. COX AND P. FAY, unpublished observation). It is probable therefore that a certain amount of the inhibitor is present even in washed particulate preparations. Further purification of the photosynthetic membranes from soluble cell components is required, both to remove inhibitory factors and also to enable the precise requirements for N_2 fixation in blue-green algae to be ascertained. From dialysis studies it is evident that some soluble small molecules are required for N_2 fixation but unless these can be removed completely, their nature can not be determined.

Elimination of O_2 during preparation and incubation of cell-free extracts from blue-green algae goes a long way towards avoiding the inhibition of N_2 fixation by oxidation. However, elimination of O_2 is difficult in light-incubated preparations where O_2 is generated photochemically[1]. It is possible that with the removal of oxidases from the particulate material the sensitivity of the N_2-fixing system to oxidation will diminish. It is unlikely that direct oxidation by O_2 in the absence of oxidases would take place.

The principal difficulty in purifying the N_2-fixing system of blue-green algae lies in the fact that it is membrane bound[1]. Also on the same membrane are sited the photosynthetic[1] as well as the respiratory[23] apparatus of the cell. Separation of an active N_2-fixing system from the membranes, if it were possible, would effect purification of the system but would also eliminate the intrinsic relationship between N_2 fixation, photosynthesis and pyruvate metabolism.

ACKNOWLEDGEMENTS

We are grateful to Dr. D. H. BROWN for valuable advice and discussions and to Professor G. E. FOGG for his interest and help. This work was supported by a grant from the Science Research Council.

REFERENCES

1 R. M. COX, P. FAY AND G. E. FOGG, *Biochim. Biophys. Acta*, 88 (1964) 208.
2 R. M. COX, Ph. D. Thesis, University of London, 1965.
3 J. E. CARNAHAN, L. E. MORTENSON, H. F. MOWER AND J. E. CASTLE, *Biochim. Biophys. Acta*, 44 (1960) 520.
4 L. E. MORTENSON, *Federation Proc.*, 25 (1966) 524.
5 D. J. D. NICHOLAS, D. J. SYLVESTER AND J. F. FOWLER, *Nature*, 189 (1961) 634.
6 D. J. D. NICHOLAS AND D. J. FISHER, *Nature*, 186 (1960) 735.
7 M. W. NIBECK, P. W. WILSON AND D. J. D. NICHOLAS, *Nature*, 200 (1963) 709.
8 R. C. BURNS AND W. A. BULEN, *Biochim. Biophys. Acta*, 105 (1965) 437.
9 W. A. BULEN, *Federation Proc.*, 25 (1966) 341.
10 R. M. COX, *Arch. Mikrobiol.*, 53 (1966) 263.
11 P. FAY AND G. E. FOGG, *Arch. Mikrobiol.*, 42 (1962) 310.
12 P. FAY, *J. Gen. Microbiol.*, 39 (1965) 11.
13 H. W. MILNER, N. S. LAWRENCE AND C. S. FRENCH, *Science*, 111 (1950) 633.
14 R. H. BURRIS AND P. W. WILSON, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. IV, Academic Press, New York, 1957, p. 355.
15 M. J. DILWORTH, D. SUBRAMANIAN, T. O. MUNSON AND R. H. BURRIS, *Biochim. Biophys. Acta*, 99 (1965) 486.

16 G. E. FOGG AND THAN-TUN, *Proc. Roy. Soc. (London), Ser. B*, 153 (1960) 111.
17 G. E. FOGG AND THAN-TUN, *Biochim. Biophys. Acta*, 30 (1958) 209.
18 H. D. COBB AND J. MYERS, *Am. J. Botany*, 51 (1964) 753.
19 F. R. WHATLEY, *Natl. Acad. Sci.-Natl. Res. Council, Publ.*, 1145 (1963) 243.
20 J. MYERS, in W. RUHLAND, *Encyclop. of Plant Physiol.*, Vol. V, Part I, Springer, Berlin, 1960, p. 211.
21 R. ULRICH, *Ann. Rev. Plant Physiol.*, 9 (1958) 385.
22 R. D. DUA AND R. H. BURRIS, *Biochim. Biophys. Acta*, 99 (1965) 504.
23 P. FAY AND R. M. COX, *Biochim. Biophys. Acta*, 126 (1966) 402.