PYRUVATE AND NITROGENASE ACTIVITY IN CELL-FREE EXTRACTS OF THE BLUE-GREEN ALGA ANABAENA CYLINDRICA

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ABSTRACT: When extracts of Anabaena cylindrica are prepared in the absence of dithionite, they catalyze pyruvate-dependent acetylene reduction, a reaction not observable in assays containing dithionite. Ferredoxin and coenzyme-A, but not NADP and ferredoxin-NADP reductase, are required for maximal pyruvate-dependent activity. These acetylene-reducing extracts do not exhibit NADP-pyruvate dehydrogenase activity. However, pyruvate:ferredoxin oxidoreductase is present at levels of activity sufficient to support the in vitro rate of pyruvate-supported acetylene reduction. These in vitro data support earlier in vivo evidence that pyruvate:ferredoxin oxidoreductase transfers electrons from pyruvate to nitrogenase in A. cylindrica.

INTRODUCTION: There is evidence from studies using intact filaments that pyruvate may supply reductant to nitrogenase in A. cylindrica (1,2,3,4). However in vitro evidence for pyruvate supporting nitrogenase activity is conflicting (5,6,7,8). The most recent detailed in vitro studies are those of Bothe (9) who could not detect pyruvate-dependent acetylene reduction by A. cylindrica preparations to which ferredoxin, NADP and ferredoxin-NADP reductase were added. He did, however, obtain activity with a system in which glucose-6-phosphate supplied electrons via an NADP-dependent glucose-6-phosphate dehydrogenase and ferredoxin-NADP reductase. The recent discovery of pyruvate ferredoxin oxidoreductase in nitrogen-fixing A. cylindrica (10, 11) and its characteristics (12) suggest that in vitro it may generate reduced ferredoxin to support nitrogen fixation but so far no direct evidence of this is available. In this paper we present

evidence of an *in vitro* stimulation of nitrogenase activity by pyruvate in *A. cylindrica* which is most likely to be due to electrons generated via pyruvate-ferredoxin oxidoreductase rather than via pyruvate dehydrogenase.

MATERIALS AND METHODS:

Organism: *Anabaena cylindrica* (Fogg's strain) (13) was grown axenically in nitrogen-free medium as described previously (11).

Preparation of extracts and acetylene reduction procedure: Cell disruptions by ultrasonication were carried out according to Haystead *et al.* (14), except that sodium dithionite was omitted, or by French pressure cell treatment at 16,000 p.s.i., again without added dithionite. A 40,000 x g x 20 min. supernatant (8) containing 5 - 12 mg protein/ml was used throughout for acetylene reduction assays. The basic reaction mixture contained in a final volume of 1.5 ml: NaHEPES pH 7.55, 30 μ moles; $MgCl_2$, 5 μ moles; ATP, 3 μ moles; creatine phosphate, 20 μ moles; creatine phosphokinase, 50 μg; $Na_2S_2O_4$, 2.5 μ moles and 1.5 to 3.0 mg protein. Other additions are as detailed in the Results and Discussion section. Reactions were carried out at 25°C in the dark. Acetylene reduction was measured as described previously (14).

Pyruvate dehydrogenase (E.C.1.2.4.1). Extracts were tested for pyruvate dehydrogenase activity using the method of Leach and Carr (15). Pyruvate-dependent NADP and NAD reductions were measured as increase in absorbance at 340 nm on a Pye Unicam SP1800 recording spectrophotometer.

Pyruvate:ferredoxin oxidoreductase (E.C.1.2.7.1). The method of Bothe and Falkenberg (10) was used. The acetohydroxamate derivative formed by pyruvate decarboxylation was measured as absorbance at 540 nm using a Pye Unicam SP600 spectrophotometer. Ferredoxin

prepared from Scenedesmus obliquus was used in the assay. Other additions are detailed in the Results and Discussion section.

Preparation and assay of ferredoxin-NADP reductase from A. cylindrica. 20 l of exponentially growing A. cylindrica were harvested by low speed centrifugation and washed with 0.05 M Tris-HCl buffer, pH 7.5. After recentrifugation, the pellet was resuspended to about 100 ml in 0.4 M Tris-HCl (pH 7.5) and the cells disrupted by passage twice through a French pressure cell at 4°C (16). The supernatant obtained after spinning at 37,500 x g for 20 min. was treated with ammonium sulphate to 40% saturation. After centrifugation at 37,500 x g for 10 min., ammonium sulphate was added to the resulting supernatant to 100% saturation. Further centrifugation yielded a pellet which was resuspended in a minimal volume of 0.025M (pH 7.5) potassium phosphate buffer and dialyzed exhaustively against the same buffer. The preparation was applied to a 25 x 2 cm column of Whatman DE52 DEAE cellulose and eluted with a linear gradient of 0.05M KCl in 0.025 M phosphate buffer. Fractions containing activity were bulked, concentrated by ultrafiltration and applied to a 40 x 2.5 cm Sephadex G-200 column, which was eluted with 0.025 M phosphate buffer, pH 7.5. The enzyme was assayed by pipetting 60 μ moles Tris-HCl, pH 7.5; 0.04 μ moles DCPIP; 0.2 μ moles $NADPH_2$ and enzyme into a cuvette and measuring the reduction of dichlorophenol-indophenol as a decrease in absorbance at 620 nm in the Unicam SP1800 spectrophotometer.

Protein determinations. Protein was measured by the method of Lowry et al. (17) using bovine serum albumin as a standard.

Various chemicals. S. obliquus ferredoxin was a kind gift from Dr. R. Powls, Department of Biochemistry, University of Liverpool. Coenzyme A, thiamine pyrophosphate and cysteine

were purchased from Sigma, St. Louis, Missouri, U.S.A. NAD, NADP, $NADPH_2$, ATP, creatine phosphate, creatine phosphokinase, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Boehringer GmbH, Mannheim, Germany. All other chemicals were bought from BDH Chemicals Ltd., Poole, U.K. at the highest purity commercially available.

RESULTS AND DISCUSSION: Table I shows that cell-free extracts of A. cylindrica reduce acetylene and, as before (9,14,18), the requirement for reductant can be met using dithionite. In the absence of dithionite pyruvate alone did not significantly stimulate acetylene reduction but relatively good rates occurred in the presence of pyruvate plus ferredoxin. Coenzyme A, which is required as an acetyl-acceptor whether enzymic pyruvate oxidation is catalysed by a nucleotide-dependent dehydrogenase (19) or by pyruvate:ferredoxin oxidoreductase (10,12) further stimulated acetylene reduction, but there was no additional enhancement when NADP and ferredoxin-NADP reductase were included in these assays. It may be noted that in order to observe the stimulation of nitrogenase activity by pyruvate it was necessary to omit dithionite which is usually added to the cell disruption medium to preserve activity. In so doing we have obtained rates of acetylene reduction of about 50% of those found with dithionite in the disruption medium. Nevertheless in foregoing maximal activity by excluding artificial reductant during enzyme preparation we obtained a stimulation which would not otherwise have been seen (8).

The possibility that sufficient endogenous NADP and ferredoxin-NADP reductase were already present in the extract to support possible pyruvate dehydrogenase activity was tested using glucose-6-phosphate dehydrogenase as reductant in the

Table I

Requirements for acetylene reduction by extracts of A. cylindrica and the effects of pyruvate and various cofactors

Reaction mixture	n moles C_2H_4 formed/ mg protein/hr	Relative rate
1. complete[a]	22.4	100
2. dithionite omitted	2.4	11
3. dithionite omitted, + pyruvate[b]	3.8	17
4. dithionite omitted, + ferredoxin[c]	3.0	13
5. dithionite omitted, + pyruvate + ferredoxin	14.2	63
6. dithionite omitted, + pyruvate + coenzyme A[d]	3.6	16
7. dithionite omitted, + pyruvate + ferredoxin + coenzyme A	20.0	89
8. dithionite omitted, + ferredoxin + pyruvate + coenzyme A + NADP[e] + ferredoxin-NADP reductase[f]	18.8	84
9. complete, using extract prepared in the presence of dithionite as in ref. (8)	46.9	

Each value is the mean of triplicates. a, see Materials and Methods; b, 15 μ moles per assay; c, 10 μ moles per assay; d, 0.1 μ mole per assay; e, 2.5 μ moles per assay; f, saturating amount per assay.

presence of NADP-dependent glucose-6-phosphate dehydrogenase. As shown previously (9), a combination of exogenous glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, ferredoxin-

NADP reductase and ferredoxin stimulated activity (Table II). However the omission of ferredoxin-NADP reductase or NADP markedly decreased the reaction rates to about the levels obtained in the absence of reductant. This indicates that ferredoxin-NADP reductase and NADP were limiting in the extracts and suggests strongly that there was no further stimulation of pyruvate-ferredoxin-CoA-dependent acetylene reduction (noted in Table I) on adding NADP plus ferredoxin-NADP reductase, because pyruvate oxidation was not catalyzed via a NADP-pyruvate dehydrogenase system. Our results, like those of Bothe (9), thus contrast with those of Smith et al. (7) who reported, without presenting data, that the addition of pyruvate together with NADP, supported acetylene reduction in

Table II

Acetylene reduction by extracts of A. cylindrica using glucose-6-phosphate as a source of reductant

	Reaction mixture	n moles C_2H_4 formed/ mg protein/hr	Relative rate
1.	complete[a]	21.6	100
2.	minus glucose-6-phosphate dehydrogenase	11.7	54
3.	minus NADP	5.4	25
4.	minus ferredoxin-NADP reductase	5.7	26
5.	minus glucose-6-phosphate	4.5	21

Each value is the mean of triplicates. a, glucose-6-phosphate dehydrogenase, 0.1 ml ; glucose-6-phosphate, 10 μ moles; NADP, 2.5 μ moles; ferredoxin, 10 μ moles; ferredoxin-NADP reductase, saturating amount per assay. Other additions as in Materials and Methods, with dithionite omitted.

Table III

Pyruvate:ferredoxin oxidoreductase activity in acetylene-reducing extracts of A. cylindrica

Assay	Specific activity u moles acetohydroxamate formed/mg protein/hr
1. complete[a]	0.642
2. minus ATP	0.392
3. minus coenzyme A	0.562
4. minus ferredoxin	0.517
5. minus pyruvate	0.006
6. + NADP[b] + ferredoxin-NADP reductase[c]	0.650
7. + NADP + ferredoxin-NADP reductase[c], minus ferredoxin	0.517

a, see Materials and Methods; b, 2.5 μ moles per assay; c, saturating amount.

extracts of A. cylindrica. Indeed all our attempts to measure pyruvate dehydrogenase either in the presence of NADP or NAD and using up to 6.4 mg protein obtained from preparations from French press treatments as well as by sonication were negative.

The data in Table III, which were obtained using the extracts which exhibited pyruvate-dependent acetylene reduction, show the presence of pyruvate:ferredoxin oxidoreductase. When measured as acetohydroxamate production maximal activity required the addition of pyruvate, coenzyme A, ATP and ferredoxin. The addition of NADP plus ferredoxin-NADP reductase (previously

shown to be limiting in these preparations, see Table II) did not alter the activity significantly, providing further evidence against the presence of pyruvate dehydrogenase activity in the extracts. On a protein basis the pyruvate:ferredoxin oxidoreductase activity was approximately 30 times greater than the rates of acetylene reduction and could thus account for the pyruvate-dependent activity noted in these extracts.

Thus, our data showing acetylene reduction *in vitro* supported by a pyruvate-dependent reaction requiring ferredoxin and coenzyme A, our failure to detect pyruvate dehydrogenase activity and our detection of pyruvate:ferredoxin oxidoreductase in the extracts suggests that electron transfer to nitrogenase occurs in our system via the latter enzyme. *In vivo* evidence supporting a role for pyruvate:ferredoxin oxidoreductase as a route of electron transfer to nitrogenase is reported elsewhere (3,4,12).

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