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PYRUVATE:FERREDOXIN OXIDOREDUCTASE AND ITS ACTIVATION BY ATP IN THE BLUE-GREEN ALGA *ANABAENA VARIABILIS*

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SUMMARY

1. The breakdown of pyruvate was examined in whole cells and cell-free extracts of the blue-green alga *Anabaena variabilis*. Decarboxylation of specifically labelled pyruvate indicated a similar metabolic route to that of acetate, although no pyruvate oxidase was present. Pyruvate:ferredoxin oxidoreductase was detected in cell-free extracts and after DEAE-cellulose treatment, addition of ferredoxin was necessary for pyruvate decarboxylation; acetyl-CoA was the first product of the reaction.

2. The formation of acetyl-CoA from pyruvate required ATP; kinetic evidence as well as experiments with [γ-^{32}P]ATP indicated that this serves as an activator and not as a substrate in the reaction. The importance of this reaction in the control of biosynthesis and nitrogen fixation is discussed.

INTRODUCTION

Although glucose is metabolised in blue-green algae *via* the glycolytic and pentose phosphate pathways[1–3] there is no information which describes the further metabolism of pyruvate in these organisms other than its conversion to acetolactate, and hence branched chain amino acids, by acetolactate synthetase[4]. The incorporation of exogenous pyruvate into several species of blue-green algae has been measured and its conversion into glutamate and proline, as well as into branched chain amino acids, observed[5]. The synthesis of the glutamate family of amino acids from pyruvate indicated that a 2-carbon moiety was formed which entered the incomplete tricarboxylic acid cycle present in these organisms[5,6].

In an organism in which carbon dioxide may serve as the sole carbon source, the origin of 2-carbon units is of especial interest. Evidence suggests that the presence of pyruvate oxidase in chloroplasts is doubtful[7] and this communication reports its absence in *Anabaena variabilis*.

COX AND FAY[8] have suggested that there exists a direct relationship between pyruvate decarboxylation and nitrogen fixation in *Anabaena cylindrica*. They suggest that this system may not be entirely analogous to the phosphoroclastic cleavage of pyruvate by the nitrogen-fixing Clostridia because the blue-green alga requires light, presumably as an ATP source, for nitrogen fixation. This paper describes the ferredoxin-linked formation of acetyl-CoA from pyruvate in extracts of *Anabaena variabilis*

and discusses the potential role, and control of the pyruvate:ferredoxin oxidoreductase in both carbon metabolism and nitrogen fixation. Some of the data presented have been briefly communicated previously[9].

METHODS

Growth and preparation of cell-free extracts

Anabaena variabilis was grown as previously described[10] on a defined mineral salts medium which was gassed with air–CO_2 (95:5, v/v). After harvesting, organisms were resuspended in 50 mM Tris–HCl buffer (pH 7.6) containing 1 mM dithiothreitol and disrupted by ultrasonic treatment in an M.S.E. 60 W 20 kc/sec sonic disintegrator for 3 periods of 45 sec at maximum output[11]. Cell wall debris and any unbroken cells were removed by centrifugation at 10000 × *g* for 15 min at 0–2°. Disruption of organisms under anaerobic conditions, which were obtained by flushing with oxygen-free nitrogen, in the presence of an –SH protecting component, led to extracts several fold more active with respect to pyruvate:ferredoxin oxidoreductase activity.

Removal of ferredoxin from cell-free extracts

Ferredoxin was removed from extracts of *A. variabilis* by the adsorption of the cell-free extracts onto DEAE-cellulose columns (2.5 cm × 3 cm) and the columns washed with a small volume of 0.1 M NaCl and 1 mM dithiothreitol in 50 mM Tris–HCl (pH 7.6). The major protein containing fractions eluted were collected and used in the enzyme assays as described. All solutions were gassed for 20 min with oxygen-free nitrogen and columns were run under an atmosphere of oxygen-free nitrogen. Introduction of aerobic conditions at any stage of the enzyme preparation resulted in lower enzymic activities.

Enzymic assays

Pyruvate oxidase and pyruvate dehydrogenase (EC 1.2.4.1). These enzymes were assayed by the methods described by KORKES[12].

By reduction of NAD^+ ($NADP^+$) in the presence of pyruvate, coenzyme A and thiamine pyrophosphate. The reaction mixture contained in μmoles; potassium phosphate buffer (pH 7.0), 200; coenzyme A, 0.05; NAD^+ ($NADP^+$), 2; cysteine, 10; thiamine pyrophosphate, 0.5; $MgCl_2$, 10 and cell-free extract (4–8 mg protein) in a total volume of 2.7 ml. The reaction was started by the addition of pyruvate (5 μmoles) to the experimental cuvette and increase in $A_{340\ nm}$ was measured.

Any CO_2 evolved or O_2 consumed was assayed manometrically. The reaction mixture had the same composition as above except 200 μmoles pyruvate were used as substrate.

The production of acetyl-CoA in the reaction mixture described above was determined by adding freshly neutralised hydroxylamine (1000 μmoles) and determining the acetohydroxamate formed as the $FeCl_3$-complex[13].

Pyruvate:ferredoxin oxidoreductase. When the enzyme was measured in untreated cell-free extracts of *A. variabilis*, the reaction mixture contained, in μmoles; potassium phosphate buffer (pH 7.0), 100; $MgCl_2$, 10; dithiothreitol, 3; coenzyme A, 0.05; ATP, 5; freshly neutralised hydroxylamine, 1000 and pyruvate, 200 in a total volume of 2.5 ml. The reaction was carried out at 34° and was started by the addition

of cell-free extract (3–10 mg protein). Acetohydroxamate was estimated by the formation of a complex with $FeCl_3$ and estimation at $A_{540\,nm}$. A standard curve relating $A_{540\,nm}$ to the concentration of acetohydroxamate $FeCl_3$ complex was constructed by the method of STADTMAN[14].

Pyruvate–ferredoxin oxidoreductase was measured in ferredoxin-free extracts of *A. variabilis* as above except that ferredoxin (0.1 mg), prepared from *A. variabilis* was included.

Hydrogen:ferredoxin oxidoreductase (Hydrogenase) (EC 1.12.1.1). This enzyme was assayed by the procedure of KORKES[15]. The assay is based on the conversion of pyruvate by lactate dehydrogenase in the presence of oxidised pyridine nucleotide, and blue-green algal extract incubated under an atmosphere of hydrogen.

Preparation of ferredoxin and NADP⁺:ferredoxin oxidoreductase. Ferredoxin and the enzyme $NADP^+$:ferredoxin oxidoreductase were prepared from *A. variabilis* as described by SUSOR AND KROGMANN[16]. The activity of the $NADP^+$–ferredoxin oxidoreductase was estimated as described by AVRON AND JAGENDORF[17].

The oxidation of [1-^{14}C]-, [2-^{14}C]- and [3-^{14}C]pyruvate by intact A. variabilis

These experiments were designed to follow the release of $^{14}CO_2$ from specifically ^{14}C-labelled pyruvate by intact *A. variabilis* in the dark. Reaction mixtures were incubated in manometer flasks and the $^{14}CO_2$ released was adsorbed by hyamine hydroxide in the centre well. The contents of the centre well were then transferred to scintillation vials and radioactivity counted in a Packard Tri-Card scintillation spectrophotometer. These experiments were essentially similar to experiments with [^{14}C]acetate and the details of the procedure can be obtained from PEARCE AND CARR[11].

Chromatographic identification of acetohydroxamate

Acetohydroxamate, formed from a product of pyruvate oxidation, was chromatographically identified as previously described[11].

Identification and estimation of [^{32}P]ATP

Esterified [^{32}P]phosphate and inorganic [^{32}P]phosphate were chromatographically identified as previously described[18]. Inorganic [^{32}P]phosphate and esterified [^{32}P]phosphate were separated by extraction of inorganic [^{32}P]phosphate as a molybdate complex into benzene:isopropanol as described by AVRON[19]. The radioactivity of the esterified [^{32}P]phosphate was determined in a Nuclear Chicago thin window gas flow counter as previously described[18].

Estimation of protein

The amount of protein present in extracts was determined colorimetrically after removal of the photosynthetic pigments by hot acid ethanol as previously described[10].

Chemicals

All chemicals were of analytical grade or of the purest commercial grade available. ATP, ADP, AMP, CoA, NAD^+, $NADP^+$, FAD, FMN and lactate dehydrogenase were purchased from C. F. Boehringer, Mannheim, Germany; acetyl phosphate,

pyruvate and spinach ferredoxin from Sigma London Chemical Co., Lettice Street, London S.W.6. Cytochrome *c* (horse heart) was purchased from Servac, Maidenhead, Bucks. and all radioactive chemicals were from The Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

The breakdown of pyruvate by *A. variabilis* was demonstrated with intact organisms by measurement of the $^{14}CO_2$ that was released on incubation with specifically labelled pyruvate. The ratio of carbon dioxide formed from [2-^{14}C]pyruvate to that released from [3-^{14}C]pyruvate was similar to that from [1-^{14}C]- and [2-^{14}C]-acetate (Fig. 1), indicating that, *in vivo*, the two compounds shared a common pathway. Pyruvate dehydrogenase activity could not be detected in extracts of *A. variabilis* by the three procedures employed although a low rate of conversion of pyruvate to acetohydroxamate (0.05 nmoles/min per mg protein) was observed. Inclusion of $NADP^+$, FMN, ferrocyanide or cytochrome *c* in the incubation mixture did not lead to their reduction not to any increase in the rate of acetohydroxamate formation.

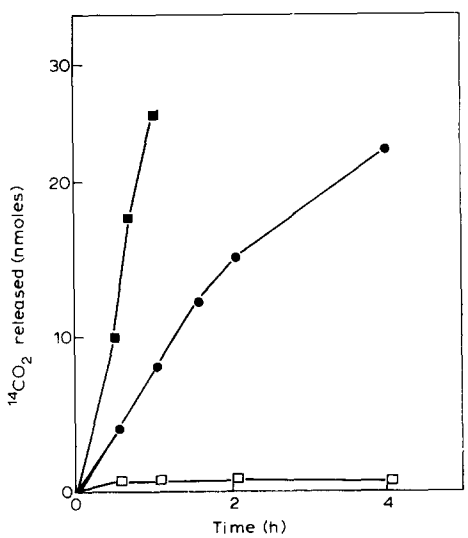


Fig. 1. The rate of release of $^{14}CO_2$ from specifically-labelled pyruvate by *Anabaena variabilis*. Each manometer flask containing 45 mg dry wt. of organism, carbon dioxide released was trapped as described in METHODS. □—□, [3-^{14}C]pyruvate; ●—●, [2-^{14}C]pyruvate; ■···■, [1-^{14}C]-pyruvate.

Incubation of pyruvate under the conditions described for the assay of pyruvate:ferredoxin oxidoreductase in extracts of *A. variabilis* containing ferredoxin led to a substantially faster rate of acetohydroxamate synthesis. Inclusion of NAD^+, thiamine pyrophosphate or coenzyme A in the reaction mixture did not affect the acetohydroxamate synthesis from pyruvate but the omission of ATP reduced the reaction 90–98 % (Table I). ATP could not be replaced by GTP.

TABLE I

FORMATION OF ACETOHYDROXAMATE FROM PYRUVATE BY EXTRACTS OF *A. variabilis*

The assays were carried out as described in METHODS; 3–9 mg of protein were used in each assay, the figures below being the mean of 3 determinations.

Substrate	*Rate of acetohydroxamate formation (nmoles/min per mg protein)*
Pyruvate	2.6
Pyruvate (ATP omitted)	0.05
Pyruvate (ATP replaced by GTP)	0.06
Pyruvate (ATP replaced by 5 μmoles ADP)	0.08
Pyruvate (ATP replaced by 5 μmoles AMP)	0.07
Acetate	4.4
Acetate (ATP omitted)	0.01
Acetate (ATP replaced by GTP)	2.0
Control experiment (extract of *A. variabilis* replaced by acetate kinase [25 units], with pyruvate as substrate)	0.2

When extracts of *A. variabilis* were replaced with a commercial preparation of acetate kinase only a small amount of acetohydroxamate was formed, indicating that there was insufficient acetate, present as an impurity in the pyruvate, to account for the levels of acetohydroxamate produced by the blue-green algal extract (Table I).

The stimulation of acetohydroxamate formation by ATP suggested that acetate may be a primary product of pyruvate oxidation as has been shown to occur in *Proteus vulgaris*[20,21]. The acetate formed from pyruvate by *A. variabilis* could then act as a substrate for acetate kinase known to be present in this organism[11]. There was,

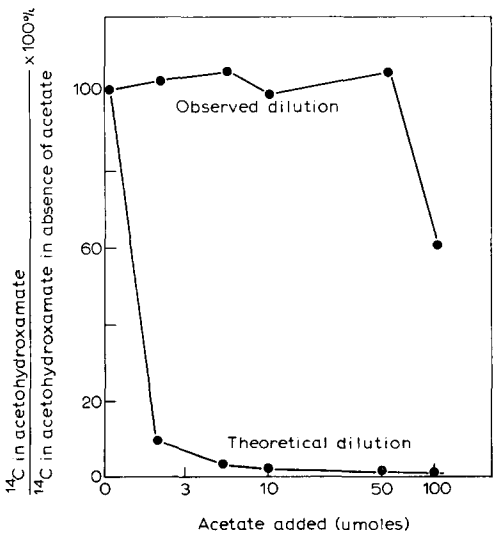


Fig. 2. Dilution by acetate of incorporation of [2-^{14}C]pyruvate into acetohydroxamate by *Anabaena variabilis*.

however, no dilution of isotope in acetohydroxamate produced from [2-^{14}C]- and [3-^{14}C]pyruvate when acetate was added to the incubation mixture (Fig. 2).

No radioactivity from [1-^{14}C]pyruvate could be traced to acetohydroxamate, neither could free acetate formation from pyruvate be detected by a procedure based on that described by STEGINK[22].

The formation of acetohydroxamate from pyruvate by extracts of *A. variabilis* occurred over a broad pH between 7.0 and 8.5 and required rather high concentrations of pyruvate for maximum activity (Fig. 3a and b). There was a small oxygen uptake associated with this process that, in the presence of pyruvate and ATP was between 0.15 and 0.23 μmoles/h per mg protein.

The dependence of acetohydroxamate formation from pyruvate by ferredoxin-free extracts of *A. variabilis* on the readdition of ferredoxin is shown in Table II.

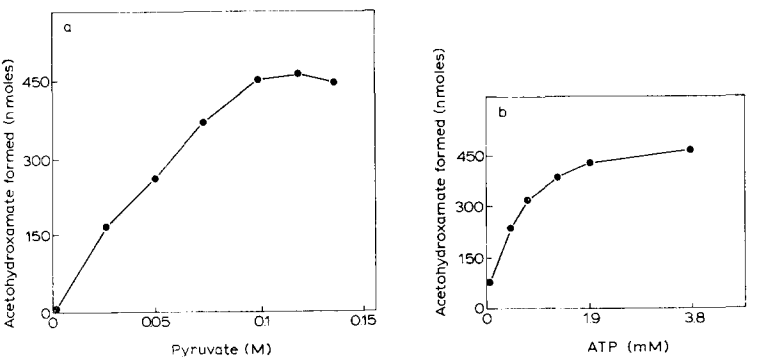


Fig. 3. Acetohydroxamate formation by extracts of *Anabaena variabilis*. Except where indicated conditions were as described in METHODS. a. Variation in pyruvate concentration, each tube contained 6.4 mg protein. b. Variation in ATP concentration, each tube contained 6.4 mg protein and 0.13 M pyruvate. Incubations were for 30 min in each case.

TABLE II

THE FORMATION OF ACETOHYDROXAMATE FROM PYRUVATE BY DEAE-CELLULOSE TREATED EXTRACTS OF *Anabaena variabilis*

The assay procedure is described under 'pyruvate:ferredoxin oxidoreductase' in the METHODS section. Cell-free extract containing 3–10 mg of protein were used in each assay.

Reaction conditions	*Rate of acetohydroxamate formation (nmoles/min per mg protein)*
Complete	1.4
ATP omitted	0.3
ATP replaced by GTP	0.3
Coenzyme A omitted	0.8
Thiamine pyrophosphate omitted	1.5
Ferredoxin omitted	0
Ferredoxin (from *A. variabilis*) replaced by ferredoxin from spinach chloroplasts	1.4
Ferredoxin replaced by 0.1 mg benzyl viologen	1.8
$MgCl_2$ omitted	0.8
$MgCl_2$ replaced by $MnCl_2$	1.2
Dithiothreitol omitted	0.9

The redox dye, benzyl viologen and spinach chloroplast ferredoxin were equally as effective as ferredoxin. The rate of conversion of pyruvate to acetohydroxamate was reduced by the omission of coenzyme A suggesting that acetyl-CoA rather than acetyl phosphate was the first product of pyruvate oxidation.

Divalent metal ions were shown to be required and the presence of an –SH protecting compound led to higher activities. Dithiothreitol (mM) was shown to be more effective than 2-mercaptoethanol (2 mM), cysteine (3 mM) or reduced glutathione (2 mM).

The time course of acetohydroxamate formation from pyruvate by ferredoxin-free extracts of *A. variabilis* in the presence and absence of ferredoxin is shown in Fig. 4. In order to confirm that ferredoxin was reduced during the oxidation of pyruvate by extracts of *A. variabilis*, ferredoxin:NADP$^+$ oxidoreductase; prepared from *A. variabilis*, and NADP$^+$ were included in the anaerobic standard incubation mixture and made anaerobic in cuvettes fitted with Thunberg stoppers. After the addition of the pyruvate, the reduction of NADP$^+$ was followed by the increase in $A_{340\,nm}$ (Table III). The rate of reduction of NADP$^+$ was of the order 0.2–0.4 nmoles/min per mg protein and was about 17–35 % of the rate of acetohydroxamate formation under similar conditions.

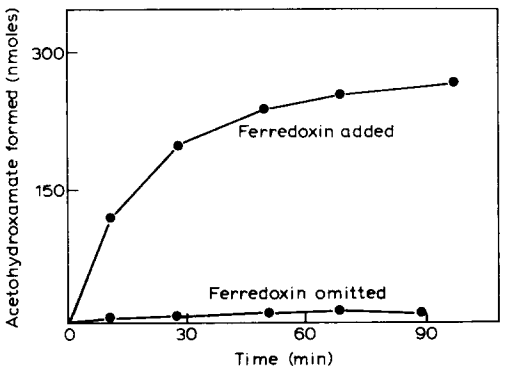


Fig. 4. Dependence upon addition of ferredoxin on acetohydroxamate formation from pyruvate by DEAE-cellulose treated extracts of *Anabaena variabilis*. The assay procedure was as described in METHODS under *Pyruvate–ferredoxin oxidoreductase*. Each tube contained 4.1 mg protein.

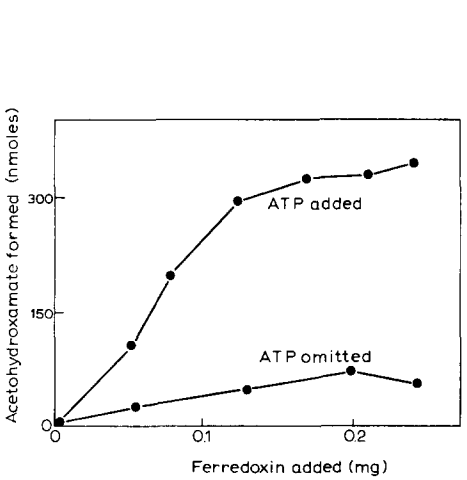
TABLE III

THE REDUCTION OF NADP$^+$ BY DEAE-CELLULOSE TREATED EXTRACTS OF *A. variabilis* IN THE PRESENCE OF PYRUVATE AND FERREDOXIN:NADP$^+$ OXIDOREDUCTASE

Assays were carried out as described and the results shown are the highest and lowest of four separate determinations containing 2–5 mg protein.

Reaction mixture	*Rate of NADP$^+$ reduction (nmoles/min per mg protein)*
Complete	0.21 –0.41
Ferredoxin omitted	0.01 –0.03
Ferredoxin:NADP$^+$ oxidoreductase omitted	0.072–0.082
Pyruvate omitted	0.03 –0.06
ATP omitted	0.05 –0.09

The role of ATP in the conversion of pyruvate to acetohydroxamate by ferredoxin-free extracts of *A. variabilis* was clarified by examination of initial reaction rates at varying ATP concentrations (Fig. 5). The degree of dependence of acetohydroxamate formation on ATP is illustrated by Fig. 6, measurable acetohydroxamate synthesis occurred in the absence of ATP indicating that ATP serves as an activator rather than a reactant in this reaction. This is further supported by the observation that there was no stimulation of the release of esterified [^{32}P]phosphate from [γ-^{32}P]-ATP by extracts of *A. variabilis* under conditions where acetohydroxamate synthesis from pyruvate was known to occur (Table IV). In contrast, when extracts of *A.*



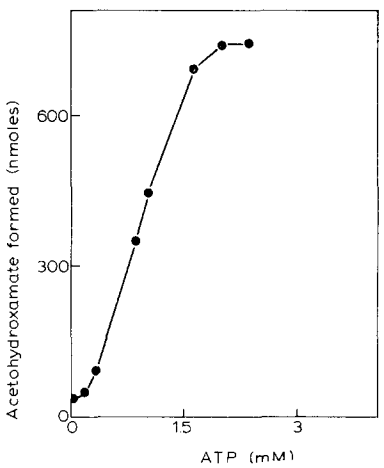


Fig. 5. Effect of ferredoxin on pyruvate: ferredoxin oxidoreductase in the presence and absence of ATP. Assays as described in METHODS; each tube contained 4.1 mg protein, and was incubated for 1 h.

Fig. 6. Effect of ATP concentration on pyruvate: ferredoxin oxidoreductase in *Anabaena variabilis*. Assayed as in METHODS except that ATP concentration was varied as shown. Each reaction contained 8.8 mg protein, and was incubated for 1 h.

TABLE IV

THE RELEASE OF [^{32}P]PHOSPHATE FROM [γ-^{32}P]ATP BY FERREDOXIN-FREE EXTRACTS OF *Anabaena variabilis* IN THE PRESENCE AND ABSENCE OF PYRUVATE

Reaction mixtures contained in μmoles; potassium phosphate buffer (pH 7.0), 100; $MgCl_2$, 10; dithiothreitol, 3; coenzyme A, 0.05; [γ-^{32}P]ATP (5 μC/μmole), 5; freshly neutralised hydroxylamine, 1000 ferredoxin (0.1 mg) and pyruvate, 200, in a total volume of 2.5 ml. The reaction was carried out at 34° and was started by the addition of cell-free extract, and [γ-^{32}P]ATP were measured as described. Results are the mean of three separate determinations carried out with 4–6 mg protein in each case.

Incubation mixture	*[^{32}P]phosphate released from [γ-^{32}P]ATP (μmoles/mg protein per 30 min)*	*Acetohydroxamate formed (μmoles/mg protein per 30 min)*
Complete	0.51	0.047
— Pyruvate	0.52	0
Pyruvate replaced by acetate	0.71	0.12
Boiled enzyme control	0	0

variabilis and [γ-^{32}P]ATP were incubated in the presence and absence of acetate, more [^{32}P]phosphate was released from [γ-^{32}P]ATP in incubation mixtures containing acetate than in incubations without this substrate. In all cases, the remaining esterified [^{32}P]phosphate was chromatographically identified as ATP.

DISCUSSION

Pyruvate:ferredoxin oxidoreductase present in extracts of *A. variabilis* forms acetyl-CoA directly from pyruvate and employs ferredoxin as the primary electron acceptor and is analogous to the clastic enzyme, typically found in the anaerobic bacteria *Clostridia* sp. The fact that the addition of non-labelled acetate did not dilute the incorporation of ^{14}C from pyruvate into acetohydroxamate, together with the failure of GTP to replace ATP in the reaction indicates that acetyl-CoA is the first product of pyruvate decarboxylation. The role of the reduced ferredoxin, produced by pyruvate:ferredoxin oxidoreductase is not known but it may be linked to ferredoxin:NADP$^+$ diaphorase, which has been demonstrated in this organism[6]. In the strain of *A. variabilis* employed nitrogen fixation does not occur although this process is widespread in the blue-green algae. It has been suggested[8] that pyruvate serves as a source of reducing power, independent of photosynthesis, for nitrogen fixation in *A. cylindrica*. The enzyme described in this paper could accomplish this process.

A role for ATP in pyruvate-supported nitrogen fixation in *Clostridium pasteurianum* by its effect on, possibly as an activator, pyruvate metabolism was suggested by DILWORTH *et al.*[23] although more recent work indicated that ADP was the effective activator[24]. In the system from *A. variabilis* described here there appeared to be a specific requirement for ATP (Table I). The addition of *S*-adenosyl-L-methionine, known to affect pyruvate metabolism in *Escherichia coli*[25], was not tested. The evidence presented here (Table IV and Fig. 6) indicates that ATP serves as an activator and not as a substrate in the pyruvate:ferredoxin oxidoreductase reaction in *A. variabilis* and allows the conversion of pyruvate to acetyl-CoA to act as an important point of metabolic control. In this organism, the decarboxylation of pyruvate is stimulated by high levels of ATP and this is consistent with the interrupted tricarboxylic acid cycle in this organism functioning in a biosynthetic, rather than oxidative, capacity. Thus, in the presence of a high intracellular level of ATP the rapid synthesis of acetyl-CoA allows biosynthesis of amino acids, fatty acids and terpenoids. Alternatively in the presence of low levels of ATP such as would occur in low light the rate of decarboxylation of pyruvate is reduced thereby reducing the rate of acetyl-CoA synthesis. In nitrogen fixing strains this control would be even more effective in that only in the presence of an adequate supply of ATP could pyruvate decarboxylation proceed and thus provide reducing potential for nitrogen fixation.

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