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ACETYLENE REDUCTION WITH PHYSIOLOGICAL ELECTRON DONORS BY EXTRACTS AND PARTICULATE FRACTIONS FROM NITROGEN-FIXING *AZOTOBACTER CHROOCOCCUM*

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SUMMARY

1. Preparations were obtained from *Azotobacter chroococcum* which reduced acetylene to ethylene using physiological electron donors instead of sodium dithionite. These preparations fell into two categories: those which required catalytic amounts of benzyl viologen for acetylene reduction and those that did not.

2. Acetylene reduction without benzyl viologen or sodium dithionite was observed only with particles that sedimented at 40000 × *g* after disrupting bacteria in the French press or with preparations obtained by disrupting bacteria protected by a mixture of defatted bovine serum albumin–Ficoll–$MgCl_2$ with liquid N_2; supernatant fractions required benzyl viologen for acetylene reduction.

3. Added ATP inhibited acetylene reduction by large particles; ATP and $MgCl_2$ were necessary for maximum acetylene reduction with bovine serum albumin-protected preparations.

4. NADH and carbon substrates acted as electron donors but H_2 did not; NAD^+ was necessary for maximum acetylene reduction with carbon substrates.

5. Anaerobic conditions were necessary for maximum acetylene reduction in all cases.

INTRODUCTION

N_2 fixation with cell-free extracts from the aerobe Azotobacter has been obtained reproducibly only with sodium dithionite as the electron source[1,2] or with a combination of H_2, hydrogenase and ferredoxin from *Clostridium pasteurianum*[3]. Attempts to obtain cell-free preparations from Azotobacter that fix N_2 using physiological electron donors have not been reproducibly successful[4], although some success was reported when cells were resuspended in bacterial medium before disrupting[5–7]; however, this could not be repeated elsewhere[3]. Azotobacter extracts contain many active dehydrogenases as well as active nitrogenase; it therefore seems likely that some step in electron transfer from carbon substrate to nitrogenase is easily damaged.

Nitrogenases catalyse the reduction of substrates other than N_2; the reduction

Abbreviation: TES, *N*-tris-[hydroxymethyl]-methyl-2-aminoethane sulfonic acid.

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of acetylene to ethylene[8–10] in particular, is a highly sensitive assay for nitrogen fixation. KLUCAS AND EVANS[12] obtained extracts from soybean nodules and from *Azotobacter vinelandii* which reduced acetylene with a physiological electron donor instead of sodium dithionite, provided benzyl viologen was present as an electron carrier; this reaction showed an absolute requirement for ATP. The present paper describes the preparation and properties of fractions from *Azotobacter chroococcum* which reduce acetylene anaerobically with physiological electron donors.

MATERIALS AND METHODS

All biochemicals were from Sigma Chemical Co. (London) and all salts from British Drug Houses, Poole, England; Ficoll, a polysaccharide, was from Pharmacia, Uppsala, Sweden. $Na_3{}^{32}PO_4$ was obtained from the Radiochemical Centre, Amersham, England; adenine nucleotide deaminase was purified from *Desulfovibrio desulfuricans*, var. *azotovorans*, Strain Berre S (National Collection of Industrial Bacteria (NCIB) No. 8388)[13]. Acetylene was made by reacting calcium carbide with water. *A. chroococcum* (NCIB No. 8003) was grown in 20-l batch cultures on nitrogen-free sucrose medium[14] at 30° for 16–20 h with forced aeration (sulfite oxidation value of 6–8 mmoles O_2/l per h (ref. 15)) and harvested during growth: harvesting started when the absorbance of the culture was 8–10 *A* units/cm at 540 mμ in an EEL colorimeter (Evans Electroselenium). These growth and harvesting conditions were necessary for subsequent subcellular fractions to show maximum acetylene reduction with physiological substrates: activity in subcellular fractions was lower if the bacteria were grown at higher or lower aeration rates or if they were harvested after growth. The *A. chroococcum* used in these experiments was a slow growing variant of Strain 8003 which produced a maximum absorbance of 16–20 $A_{540\ m\mu}$ units/cm after 24 h.

Preparations from A. chroococcum

Acetylene reduction was obtained with preparations from *A. chroococcum* using three types of electron-donating systems; (i) the sodium dithionite-dependent system, corresponding to that described by KELLY[2]; (ii) a benzyl viologen-dependent system analogous to that described by KLUCAS AND EVANS[12]; (iii) a physiological system which reduced acetylene without any artificial electron donor or electron carrier. The terms dithionite-dependent, benzyl viologen-dependent and physiological will be used to describe these electron-donating systems throughout this paper.

The following fractionation procedure was used to obtain various acetylene-reducing preparations from *A. chroococcum*.

A thick suspension of bacteria in water (40 g wet wt./50 ml final volume) was adjusted to pH 7.8 with 40% KOH and disrupted in a French pressure cell from 8000 lb/inch². The homogenate was adjusted to pH 7.4 and centrifuged at 40000 × *g* for 20 min to yield a supernatant (S_1) and a sediment of three layers: the top two layers were removed, resuspended in 0.1 M Tris (pH 7.4) (10 ml) and recentrifuged. The upper two-thirds of the sediment was suspended in the same buffer, to give a final volume of 10 ml containing approx. 60 mg protein/ml: this was called the 'large particle' fraction; the supernatant (S_1) was centrifuged at 200000 × *g* for 2 h to yield a sediment referred to as 'small particles'; these were resuspended in Tris to give 30 mg protein/ml. All preparations were made at 0–5°.

Physiological acetylene reduction by large particles was low and variable. In an alternative procedure which yielded more active acetylene-reducing systems, the cells were suspended in a mixture of defatted bovine serum albumin (1 %) + Ficoll (2.5 %) + $MgCl_2$ (0.05 M) in 0.05 M Tris buffer (pH 7.7), and disrupted by adding drop by drop to liquid N_2 and thawing; the resulting suspension was used directly in assays. The protecting mixture of bovine serum albumin–Ficoll–$MgCl_2$ was a modification of that used to protect oxidative phosphorylating preparations from adipose tissue[16]; such preparations from *A. chroococcum* are referred to here as bovine serum albumin-protected preparations.

Enzyme assays

Acetylene reduction assays were run at 30° for up to 1 h in shaken 6.5-ml bottles containing 150 μmoles Tris buffer (pH 7.4, 7.7 or 8.0) with approx. 6 mg of protein, 10 μmoles NADH or an alternative electron donor (usually 50 μmoles glucose 6-phosphate and 0.5 μmole NAD^+) in a final volume of 1.5 ml, under Ar containing 4 % acetylene. ATP was added as ATP alone or as ATP *plus* an ATP-generating system (ATP, 5 μmoles; $MgCl_2$, 20 μmoles; phosphocreatine, 20 μmoles; creatine kinase, 50 μg). Reactions were stopped with 40 % KOH (0.1 ml) and ethylene in a 1- or 2-ml gas sample was estimated at 60° in a Pye 104 gas chromatograph with a 5-ft Porapak R column of 4-mm internal diameter, using N_2 (50 ml/min) as the carrier gas and with a flame ionisation detector head.

RESULTS

Activity of acetylene-reducing systems in A. chroococcum preparations

Table I shows the acetylene-reducing activities of various preparations: the high acetylene reduction by large particles with sodium dithionite suggests that a significant proportion of the nitrogenase was bound to these particles. Physiological or benzyl viologen-dependent acetylene reduction was very small compared with that with dithionite; the supernatant had no detectable physiological activity. However, small particles (obtained from S_1, see MATERIALS AND METHODS) showed some activity with a physiological electron donor alone. The bovine serum albumin-protected preparations yielded low dithionite- and benzyl viologen-dependent activity although displaying the greatest relative physiological activity. All acetylene-reducing activities were ATP-dependent except the physiological system in large particles; this matter is discussed below.

Viability counts in A. chroococcum preparations

Bovine serum albumin-protected preparations contained less than 1 % and large particles less than 0.1 % viable organisms compared with growing *A. chroococcum*. Representative values were: bovine serum albumin-protected preparations: $2 \cdot 10^6$/ml; large particles: $1.5 \cdot 10^5$/ml; growing bacteria $3 \cdot 10^8$/ml.

Stability during storage

Dithionite-dependent and benzyl viologen-dependent acetylene reduction in all bacterial preparations were stable for 2–3 weeks and 4–7 days, respectively, at −20° before slowly deteriorating. Physiological acetylene reduction deteriorated

TABLE I

ACETYLENE REDUCTION BY PREPARATIONS FROM *A. chroococcum*

Conditions: Each preparation was tested anaerobically in 0.07 M Tris buffer at optimum pH (see text) under Ar *plus* 4 % acetylene. Energy was added as ATP *plus* an ATP-generating system as described in the text or as ATP alone (20 μmoles) with the bovine serum albumin-protected preparations. Protein concentrations: large particles, 6.2 mg; supernatant S_1, 5.5 mg; small particles, 9.2 mg; bovine serum albumin-protected preparations, 7.5 mg. Figures for the ethylene produced were compiled from a linear rate of acetylene reduction.

	nmoles ethylene produced/h							
	Large particles		*Supernatant S_1*		*Small particles*		*Bovine serum albumin-protected preparations*	
ATP, creatine phosphate *plus* creatine phosphokinase	+	−	+	−	+	−	+	−
Sodium dithionite (40 μmoles)	3850	0.5	7320	0.9	6390	0.7	520	0
Glucose 6-phosphate (50 μmoles) *plus* benzyl viologen	273	17	287	0.8	56	0	73	7.1
Glucose 6-phosphate (50 μmoles)	14	19	0	0	7.3	4.1	41	8.3

rapidly at −20°: that of large particles within 3 days and that of small particles and bovine serum albumin-protected preparations overnight. These preparations were usually stored in liquid N_2. On the other hand, harvested cells could be stored for 2 weeks at −20° and still yield large particles which possessed physiological acetylene-reducing activity.

The effect of benzyl viologen and its homologues

Benzyl viologen stimulated acetylene reduction at low concentrations and inhibited at higher levels; the optimum concentration was between 60 and 90 μM. Approx. 4 mg of protein/ml was used for routine assay with 80 μM benzyl viologen. Methyl viologen substituted for benzyl viologen but was only half as efficient; 1,1'-trimethylene dipyridylium 2,2'-dibromide ('triquat') was ineffective.

pH optima of the acetylene-reducing systems

The pH optimum of the acetylene-reducing preparations of all kinds was, with two exceptions, 7.9 ± 0.2 in Tris or *N*-tris-[hydroxymethyl]-methyl-2-aminoethane sulfonic acid (TES) buffers. The benzyl viologen-dependent acetylene reduction with the supernatant S_1 had a pH optimum of 7.4 in Tris or TES buffer; the physiological acetylene reduction in large particles using either NADH, glucose 6-phosphate, sodium pyruvate or sodium succinate as electron donor, had a pH optimum in TES of 7.4 and in Tris a narrow optimum at pH 7.7. Whole cells that were resuspended in buffer after harvesting but were not disrupted had no clearly defined pH optimum for physiological acetylene reduction.

Substrate requirements for physiological acetylene reduction

Physiological acetylene reduction in large particles was enhanced by NADH or by catalytic amounts of NAD^+ (Fig. 1); bovine serum albumin-protected preparations did not consistently respond to NADH above a blank value; nevertheless, NADH was added routinely to assays. Physiological acetylene reduction responded to other metabolites only if large particles were first dialysed overnight against 25 mM Tris (pH 7.4) to reduce the blank value. NADH and glucose 6-phosphate were the most efficient electron donors for both physiological and benzyl viologen-dependent acetylene reduction; the highest levels of dehydrogenase activity (determined by the rate of reduction of tetrazolium chloride[17]) was also associated with these substrates. Other effective substrates were glucose, sucrose, fructose and the sodium salts of succinic, pyruvic (*plus* CoA and thiamine pyrophosphate), malic, β-hydroxybutyric and 6-phosphogluconic acids; NAD^+ was necessary for maximum acetylene-reducing activity. Sodium citrate or isocitrate supported physiological and benzyl viologen-dependent acetylene reduction in large particles but they inhibited benzyl viologen-dependent reduction in the supernatant: isocitrate (30 mM) caused 95% inhibition in the presence of glucose 6-phosphate. H_2 (10%) was ineffective as a substrate for acetylene reduction.

O_2 sensitivity

Benzyl viologen-dependent and sodium dithionite-dependent acetylene reduction in all types of preparations was prevented by O_2. On the other hand physiological acetylene-reducing activity by bovine serum albumin-protected preparations was observed under O_2 although it was markedly inhibited by O_2: 95% inhibition occurred at a pO_2 of 0.05 atm (Fig. 2). Physiological acetylene reduction by large particles was more tolerant of O_2, though marked inhibition occurred at pO_2 of

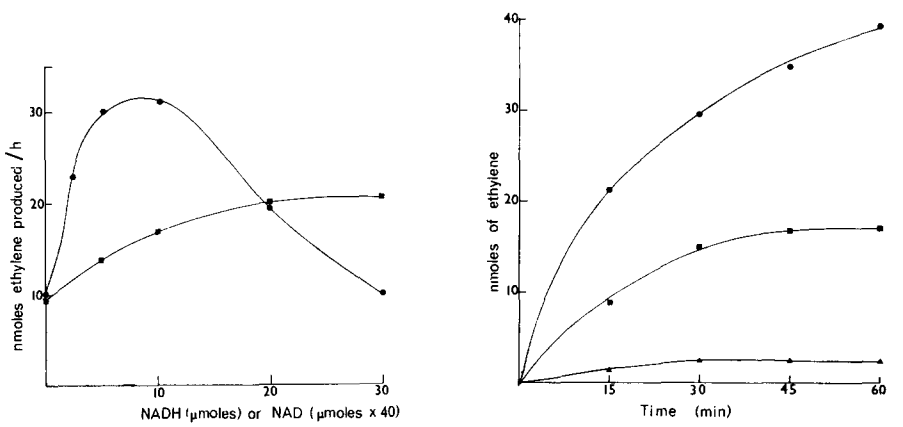

Fig. 1. Effect of NADH and NAD^+ concentration on physiological acetylene reduction by large particles from *A. chroococcum*. Conditions: as described in the text using 0.1 M Tris buffer (pH 7.7) with glucose 6-phosphate (50 μmoles) as electron donor in the experiment with NAD^+ and large particles (6.2 mg) protein. ●—●, NADH; ■—■, NAD^+.

Fig. 2. Effect of O_2 on physiological acetylene reduction by bovine serum albumin-protected preparations from *A. chroococcum*. Conditions: as described in the text using glucose 6-phosphate (50 μmoles) and NAD^+ (0.2 μmole) with bovine serum albumin-protected preparations (8.7 mg protein) under Ar/O_2 mixtures. pO_2: ●—●, zero; ■—■, 0.025; ▲—▲, 0.05 atm.

0.3 atm. There was no lag phase with either system under O_2; these findings support the contention that N_2 fixation is an anaerobic process even in aerobic N_2-fixing organisms[18] but they suggested that some of the particle-bound nitrogenase was protected from inhibition or damage by O_2, possibly by mechanisms similar to those in whole cells[19].

Role of adenine nucleotides in the physiological system

Bovine serum albumin-protected preparations required ATP or ADP for maximum physiological acetylene reduction although high levels of ATP or ADP inhibited (Fig. 3). AMP also stimulated acetylene reduction though much less effectively than ADP or ATP (Fig. 3): the best stimulation obtained with AMP was half that with ADP (mole/mole). Bovine serum albumin-protected preparations esterified P_i under anaerobic conditions; this esterification was enhanced by ATP or ADP but not by AMP. The possibility exists that P_i was esterified as a result of electron transfer to nitrogenase; however, bovine serum albumin-protected prepara-

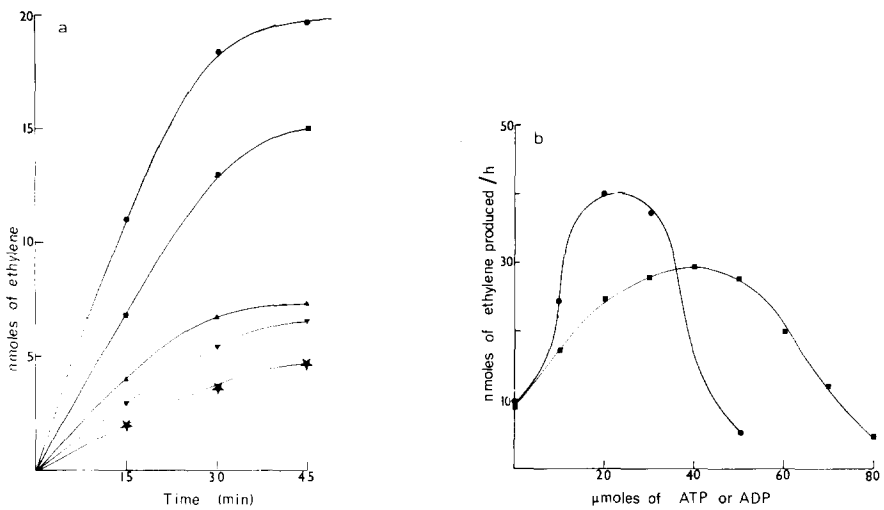

Fig. 3. a. Effect of nucleotides on physiological acetylene reduction by bovine serum albumin-protected preparations of *A. chroococcum*. Conditions: as described in the text using 20 μmoles of nucleotides, NADH (10 μmoles), *A. chroococcum* protein (8.5 mg), $MgCl_2$ (50 μmoles) in 0.1 M Tris (pH 7.7) (1.5 ml final volume) under Ar containing 4% acetylene. Additions: ●—●, ATP; ■—■, ADP; ▲—▲, AMP; ▼—▼, ATP *plus* an ATP-generating system; ★—★, nothing. b. Effect of ATP and ADP on physiological acetylene reduction by bovine serum albumin-protected *A. chroococcum* preparations. Conditions: as described in the text using *A. chroococcum* protein (7.9 mg), $MgCl_2$ (50 μmoles) and NADH (10 μmoles) in 0.1 M Tris buffer (pH 7.7) (1.5 ml). ■—■, ADP; ●—●, ATP.

tions from urea-grown *A. chroococcum*, which contained no physiological acetylene-reducing activity, esterified P_i as rapidly as did acetylene-reducing preparations.

In contrast to the other preparations, the physiological activity in large particles was inhibited by ATP, ADP or by ATP *plus* an ATP-generating system: generally ATP or ADP (3 mM) inhibited 20–40% of the acetylene-reducing activity while ATP *plus* an ATP-generating system inhibited 60–80%. Nevertheless, the following evidence suggests that ATP might be necessary: (a) the large particles

esterified P_i under anaerobic conditions; (b) sodium phosphate (30 mM) and sodium arsenate (30 mM) inhibited acetylene reduction; (c) P_i was released during acetylene reduction; both this release of P_i and acetylene reduction were inhibited by 0.1 mM sodium dithionite. However, neither the addition of an 'ATP-trap' consisting of glucose *plus* glucose hexokinase nor Desulfovibrio adenine nucleotide deaminase (which converts adenine nucleotides into inosine nucleotides[13]; ITP does not support acetylene reduction) affected acetylene reduction.

The effect of Mg^{2+}

In bovine serum albumin-protected preparations added $MgCl_2$ was necessary for maximum acetylene reduction with or without added nucleotides but it was inhibitory above an optimum level (Fig. 4). In large particles high levels of $MgCl_2$ (>30 mM) inhibited acetylene reduction but no stimulation was observed at low $MgCl_2$ concentrations. No significant stimulation occurred with ATP or ADP at any concentration of $MgCl_2$.

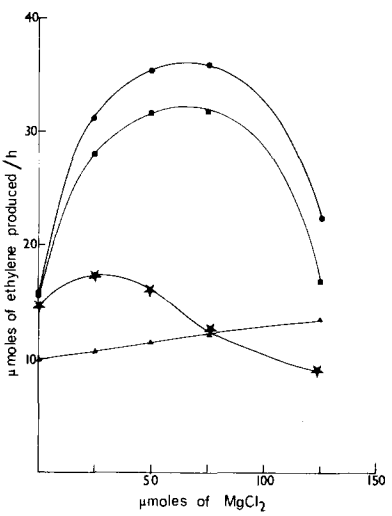

Fig. 4. Effect of $MgCl_2$ on physiological acetylene reduction by bovine serum albumin-protected preparations of *A. chroococcum*. Conditions: as described in the text using nucleotides (20 μmoles), NADH (10 μmoles) with *A. chroococcum* protein (8.5) in 0.1 M Tris buffer (pH 7.7). ●—●, ATP; ■—■, ADP; ▲—▲, ATP *plus* an ATP-generating system; ★—★, control.

Effect of inhibitors of N_2 fixation on acetylene reduction

The inhibitors of N_2 fixation CO, CN^- or N_3^- inhibited physiological acetylene reduction with either glucose 6-phosphate or NADH as the electron donor: 90% inhibition occurred with 10% CO, 6 mM CN^- or 20 mM N_3^-. Dithionite-dependent acetylene reduction was similarly inhibited.

Physiological acetylene-reducing systems from other N_2-fixing aerobes

A. vinelandii and *Mycobacterium flavum* 301 yielded large particles that contained physiological acetylene-reducing activity; urea-grown *A. chroococcum* yielded no such system.

DISCUSSION

The evidence in this paper suggests that physiological electron transport to nitrogenase in *A. chroococcum* is associated with particles which may be membrane bound; Azotobacter possess an extensive convoluted membrane (see ref. 20). It is difficult to obtain particulate preparations entirely free from whole bacteria but four lines of evidence indicate that the physiological acetylene-reducing system described here was cell-free: (i) the unstable nature of the activity compared with that with whole cells; (ii) the need for added NAD^+ or NADH for maximum acetylene reduction and the effects of ATP and ADP; (iii) anaerobic test conditions gave best acetylene reduction (growing *A. chroococcum* does not reduce acetylene anaerobically); (iv) the large particles showed a narrow pH optimum for physiological acetylene reduction in Tris buffer, whereas non-disrupted cells showed no clear pH optimum. In addition, unpublished evidence from this laboratory showed that glucose 6-phosphate, one of the best substrates for acetylene reduction by large particles, did not support acetylene reduction as well as did glucose or sucrose in growing cells.

The contrary effects of ATP or ADP on acetylene reduction by large particles and bovine serum albumin-protected preparations are not easily explained. Even if ATP production was not rate-limiting for acetylene reduction by large particles this is no reason why relatively small amounts of ATP or ADP should inhibit. It is possible that electron transport to nitrogenase in bovine serum albumin-protected preparations is analogous to electron transport to O_2 in 'tightly coupled' mitochondria[21,22]: namely, that ADP stimulates electron transport by being converted to ATP. By the same analogy a high steady-state level of ATP in the presence of an ATP-generating system would depress electron transport to nitrogenase. Since ATP is also necessary for N_2 fixation the degree of inhibition by ATP *plus* an ATP-generating system would depend on how 'tightly coupled' the system was: perhaps a high concentration of Mg^{2+} caused some 'uncoupling' and allowed ATP *plus* an ATP-generating system to stimulate acetylene reduction. A system like this in large particles would also account for the inhibition of acetylene reduction by ATP *plus* an ATP-generating system but not for the inhibition by ATP or ADP alone.

Physiological acetylene reduction by all active preparations took place on a small scale compared with the dithionite-dependent system. However, the two systems were essentially similar in their response to inhibitors of N_2 fixation, in the anaerobic nature of the process, in the energy requirement, and their absence from urea-grown *A. chroococcum*; these properties emphasise their dependence upon the presence of nitrogenase.

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