EFFECT OF NON-HAEM IRON PROTEINS AND CYTOCHROME *C* FROM *AZOTOBACTER* UPON THE ACTIVITY AND OXYGEN SENSITIVITY OF *AZOBACTER* NITROGENASE

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1. Introduction

There is substantial evidence that nitrogen fixation is an anaerobic process even in aerobic organisms [1, 2] and that nitrogenase from all sources contains two non-haem iron proteins, one of which is extremely oxygen sensitive [3]. Nitrogenase in crude extracts from *Azotobacter chroococcum* is only slowly damaged by exposure to pure oxygen although purified fraction 2 from the same organism is destroyed by a 10 min exposure to air at room temperature [3]. Postgate and his colleagues [4, 5] have suggested that nitrogenase in crude extracts of *A. chroococcum* is conformationally protected against oxygen damage in a state analogous to the 'switched off' state in whole cells [4–6]; this conformational protection may occur by association with other proteins. Recently, Arnon and his colleagues observed that two non-haem iron proteins, azotoflavin [7] and *Azotobacter* ferredoxin [8] supported nitrogen fixation in particulate preparations from *A. vinelandii* when coupled to spinach chloroplasts as an electron source. They [8] suggested that these proteins were electron carriers in the physiological electron transport chain to nitrogenase. If this is so, and these proteins are part of an electron transport chain to nitrogenase then, by analogy with electron transport to oxygen, they might be physically associated with nitrogenase *in vivo* and, by this association, protect it from oxygen damage.

The present paper confirms that proteins similar to azotoflavin and *Azotobacter* ferredoxin are present in *A. chroococcum* and described their ability, and that of other iron proteins from *A. chroococcum*, to stimulate nitrogenase activity. It also reports their effect upon oxygen sensitivity.

2. Materials and methods

Azotobacter chroococcum (N.C.I.B. 8003) was cultured and harvested as described previously [9] and stored in liquid nitrogen. Biochemicals were obtained from Sigma Chemical Co. (London) Ltd., and salts from British Drug Houses, Poole, Dorset.

2.1. *Assay of nitrogenase*

Nitrogenase was assayed by acetylene reduction [10–12] as described previously [9] with sodium dithionite as the reductant. Reactions were stopped after 20 min with 40% KOH (0.1 ml).

2.2. *Assay of NADH dehydrogenase*

NADH dehydrogenase was measured by the rate of reduction of benzyl viologen [13] or triphenyl tetrazolium bromide [14].

2.3. *Assay of oxygen sensitivity*

This was done in two ways: (a) by exposing nitrogenase to oxygen or air as described by Kelly [3]; (b) by shaking in buffer at assay concentrations (0.75–3 mg protein/ml) at 6 to 8° in air. Anaerobic controls were run under Ar simultaneously, and ATP-generating system and sodium dithionite were then added and the rate of acetylene reduction was measured anaerobically.

2.4. *Purification of proteins*

Nitrogenase. A crude supernatant (S_1, containing 70–90 mg protein/ml) obtained from *A. chroococcum* as described previously [9] was chromatographed anaerobically on DEAE -32 cellulose essentially as described by Kelly et al. [15] except that, after elution with 150 mM NaCl, both components of nitrogenase were eluted as a single fraction with 90 mM $MgCl_2$. The column yielded several distinct fractions in the following order: a NADH dehydrogenase, a cytochrome containing fraction, a yellow protein, nitrogenase, a blue protein and a second yellow protein. All fractions were dialysed anaerobically overnight to remove sodium dithionite and stored in liquid nitrogen. Subsequent tests for acetylene reduction showed that nitrogenase was completely free from residual dithionite.

Azotoflavin, azotobacter ferredoxin and cytochrome c_4 and c_5. These proteins were isolated from *A. chroococcum* as described for *A. vinelandii* [8, 16]. Disc gel electrophoresis at pH 7.4 indicated that each protein was > 90% pure and absorption spectra were similar to those reported for the *A. vinelandii* proteins [7, 8, 17]. A third, relatively impure, non-haem iron protein was obtained during this procedure [8] which had an absorption spectrum similar to that of 'ferridin' [18].

2.5. *Isolation of a heat-stable, dialysable factor which restored acetylene reduction*

Cell membranes and debris from *A. chroococcum* (200 g wet wt.) were boiled for 15 min in 3 volumes of water, filtered, and the filtrate was concentrated to 30 ml by rotary evaporation at 50°. After centrifuging to remove insoluble material, the supernatant was passed successively through the following columns: florisil (10 × 1 cm), Dowex 50 in the H^+ form (18 × 1 cm). The pH of the eluate was then adjusted to neutrality and it was passed through acid-washed charcoal (4 × 1 cm) and finally a mixed resin Bio-Rad AG 11A8 (50 × 1 cm). The factor passed unimpeded through all these columns and freeze-dried to a highly deliquescent white powder. Chemical tests indicated amino-sugar properties but NMR spectroscopy showed the absence of ring protons, characteristic of sugars. The molecular weight was approximately 500.

Partial purification of NADH dehydrogenase. The NADH dehydrogenase fraction obtained during nitrogenase purification contained no nitrogenase activity. It was solubilised by standing overnight in 1% sodium deoxycholate at 4° and then precipitated with ammonium sulfate (35 to 45%) at pH 7.0. The precipitate was dissolved in 50 mM tris buffer and dialysed overnight at 4° against the same buffer to remove residual ammonium sulfate. Purification was 10 to 15 fold.

3. Results

3.1. *Properties of nitrogenase*

The nitrogenase, purified as described, will be called A_{1+2} to distinguish it from fraction 1 (A_1) and fraction 2 (A_2) and nitrogenase in crude extracts (AS_1). The specific activity of A_{1+2} was 5 to 10 times that of AS_1 but, more significantly, it contained no NADH or succinic dehydrogenase, nor cytochromes or flavins. Despite the absence of these proteins, which are normally present in particulate nitrogenase obtained by centrifuging AS_1, A_{1+2} sedimented after 4 hr at 200,000 *g*. The rate of sedimentation depended on concentration: at 20 mg protein/ml, 18% protein remained in the supernatant; at 3 mg protein/ml 70% remained in the supernatant and the nitrogenase activity remaining in the sediment decreased accordingly.

3.2. *Stimulation of acetylene-reducing activity by added proteins*

Nitrogenase activity falls off and is not proportional to protein concentration below 1 mg/ml [19]. At low nitrogenase concentrations (70 mg/ml) acetylene reduction was stimulated or apparently restored by the dialysable factor (fig. 1a) or by crude or soluble NADH dehydrogenase, cytochrome c_4, azotoflavin, ferredoxin or the third non-haem iron protein, 'ferridin' (fig. 1b). These proteins had no nitrogenase activity of their own. At higher concentrations of A_{1+2} no stimulation occurred; on the contrary, addition of these proteins caused slight inhibition of activity. Optimal levels of activity were obtained at the following concentrations (mg/ml of assay): ferredoxin 0.26; azotoflavin 0.3; 'ferridin' 1; cytochrome c_4 0.2; dialysable factor 2; the factor was the best stimulant with dialysed AS_1, (undialysed AS_1 was unaffected), the proteins caused only 50 to 100% stimulation; cytochrome c_4 was best with A_{1+2}. When optimal amounts of these stimulants were ad-

ded together no obvious augmentation of activity occurred.

3.3. *Protection of nitrogenase against oxygen damage*

Effect of purity of nitrogenase fraction. Using a comparable technique to that of Kelly [3], A_{1+2} lost all activity in oxygen after 10 min and 80% activity in air after 30 min; AS_1 was unaffected by this treatment and A_2 lost all activity in air after 10 min [3].

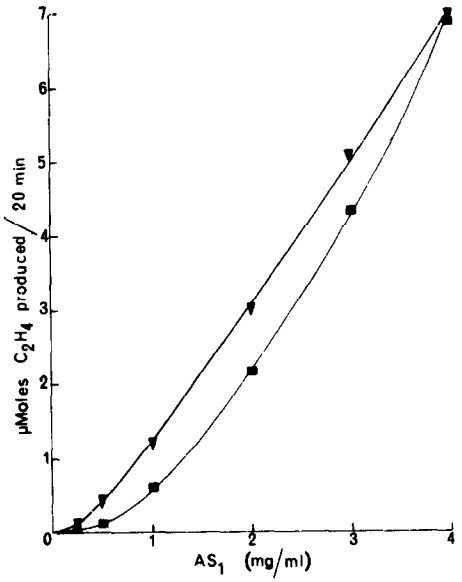
Effect of nitrogenase concentration. Different concentrations of A_{1+2} were exposed to air in assay bottles and the acetylene reducing ability was then measured. The percent loss of activity is shown in fig. 2. Alternatively, different concentrations of A_{1+2} were exposed to air and then aliquots were transferred to assay bottles to give the same concentration of A_{1+2} in each assay. Similar effects were observed, namely, as the concentration of A_{1+2} increased it became, apparently, less susceptible to oxygen damage: at 1 mg/ml less than 3% activity remained after 20 min exposure; at 4 mg/ml almost 50% remained.

3.4. *Effect of other proteins upon oxygen sensitivity*

Either crude or soluble NADH dehydrogenase offered complete protection to A_{1+2} against oxygen damage (fig. 3). Addition of any other single protein: cytochrome c_4, ferredoxin, azotoflavin or the dialysable factor failed to protect against oxygen. There was some indication that these factors increased the oxygen sensitivity of A_{1+2}, but since this sensitivity varied sufficiently to make this observation doubtful, these proteins were tested with AS_1. Each of these proteins, at the concentration giving optimal stimulation, caused a distinct increase in the oxygen sensitivity of AS_1; cytochrome c_4 and the dialysable factor were the most effective (fig. 4).

4. Discussion

There are four possible mechanisms to account for the relative inactivity of nitrogenase at low concentrations: (1) inhibitors present in reagents; (2) dissociation of a co-factor from nitrogenase; (3) ag-



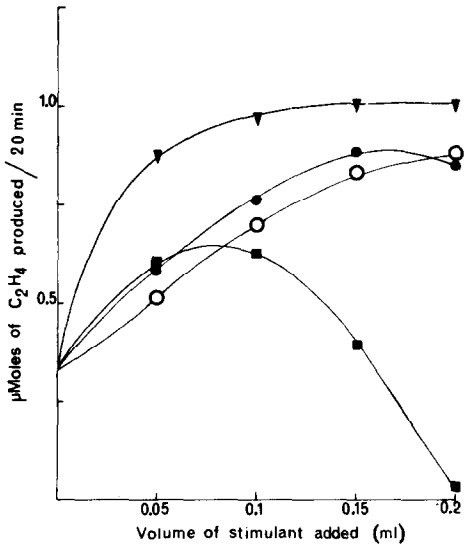


Fig. 1a. Effect of dialysable factor upon the rate of acetylene reduction by dialysed crude extract (AS_1). Conditions as described in the text using tris buffer (50 µmoles). ■—■ control; ▼—▼ control plus factor (2 mg/ml).

Fig. 1b. Effect of various proteins or the dialysable factor upon the rate of acetylene reduction by A_{1+2} (0.67 mg/ml) in HEPES buffer (50 µmoles). Additions (mg/ml): ▼—▼ cytochrome c_4 (1) or soluble NADH dehydrogenase (3.5); ●—● ferredoxin (1.75); ○—○ azotoflavin (1.5); ■—■ dialysable factor (40 mg).

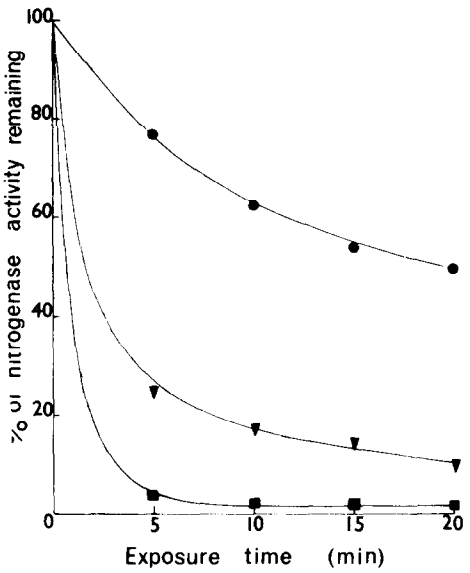


Fig. 2. Effect of concentration of A_{1+2} upon sensitivity towards oxygen damage. Conditions as described in the text (b) using HEPES buffer (50 μmoles: ■—■ 1 mg A_{1+2}/ml; ▼—▼ 2 mg A_{1+2}/ml; ●—● 4 mg A_{1+2}/ml.

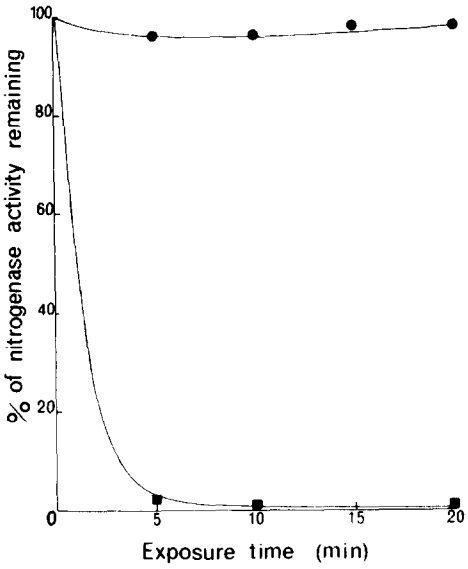


Fig. 3. Effect of various proteins upon oxygen sensitivity of A_{1+2} (1 mg/ml). Additions present at optimum concentration for stimulation. ●—● A_{1+2} plus crude (0.8 mg/ml) or soluble (0.57 mg/ml) NADH dehydrogenase; ■—■ A_{1+2} or A_{1+2} plus cytochrome c_4, ferredoxin, azotoflavin or the dialysable factor.

gregation of A_{1+2} into an inactive complex; (4) disaggregation of A_{1+2} or dissociation of A_1 from A_2 followed by either non-specific recombination or no recombination at all. (1) is unlikely because unpublished data in this laboratory has shown that stimulation by the dialysable factor occurs in a wide variety of chemical environments: tris, TES* or HEPES buffers, in the absence of ATP-generating system, at different concentrations of ATP, in the benzyl viologen system [9] where NADH and benzyl viologen replace sodium dithionite and also when fresh, glass-distilled water was used. (2) is unlikely since no co-factor, other than ATP or Mg^{2+} has been reported for nitrogenase and these are always present in the assay. (3) is also unlikely because A_{1+2} sediments less readily in dilute solutions. For this reason the fourth possibility is the most likely explanation. Stimulation of nitrogenase activity by the dialysable factor or various proteins may be because they are able to prevent dissociation or disaggregation or re-establish the correct association for optimum activity. Because of the chemical diversity of these various active factors it seems unlikely that this effect reflects enzymic or electron-transporting properties but it may reflect physical association with the nitrogenase *in vivo*.

From the results presented here, oxygen sensitivity of A_{1+2} is modified by protein concentration or by association with other proteins: it is less sensitive to oxygen damage at high than at low protein concentration and association with crude or partly purified and soluble NADH dehydrogenase protects against O_2 damage. Association with proteins, other than NADH dehydrogenase, which stimulate the rate of acetylene reduction renders nitrogenase more sensitive to oxygen damage. To explain these observations one must postulate two conditions in which nitrogenase can be damaged by oxygen. Firstly, a state in which A_1 dissociates from A_2 and A_2 becomes oxygen sensitive;

* *Abbreviations:*
HEPES: potassium *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid.
TES : potassium *N*-tris[hydroxymethyl]-methyl-2-aminoethane-sulphonic acid.

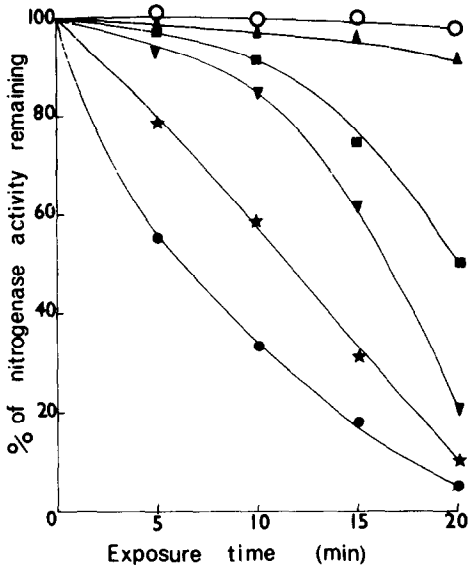


Fig. 4. Effect of various proteins upon oxygen sensitivity of AS_1. Conditions as described in the text using HEPES buffer (50 μmoles): o—o dialysed AS_1 (1.6 mg/ml); ▲—▲ undialysed AS_1, (2 mg/ml); ■—■ dialysed AS_1 plus ferredoxin or azotoflavin; ▼—▼ dialysed AS_1 plus dialysable factor (2 mg/ml); ★—★ dialysed AS_1 plus dialysable factor (4 mg/ml); ●—● dialysed AS_1 plus cytochrome c_4 (0.2 mg/ml).

this state might prevail at low concentrations of A_{1+2}. Secondly, a state in which the conformation of nitrogenase is such that the oxygen sensitive site is exposed. This may be similar to the 'switched on' state postulated by Dalton and Postgate [4] in which the nitrogenase is functional; normally nitrogenase in crude extracts is not oxygen sensitive and is 'switch off' [4]. Increased oxygen sensitivity due to adding the various proteins as described here might be because they induce the 'switched on' state. This would have to be in addition to preventing the dissociation or disaggregation of A_{1+2} for if these factors increased oxygen sensitivity by increasing dissociation they should also decrease, rather than increase, the rate of acetylene reduction.

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