THE EFFECT OF PHOSPHATE AND OXYGEN ON THE BIOCHEMISTRY AND ULTRASTRUCTURE OF *Azotobacter chroococcum*

by

PAK CHOW LEUNG

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of Master of Science in the Department of Microbiology in August, 1979.

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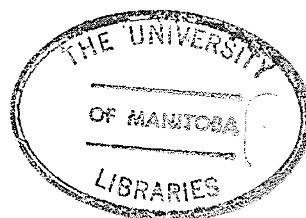

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to my family and my friends

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Abstract

Nitrogen-fixing Azotobacter chroococcum ATCC 7493 was grown in a chemostat with vortex stirring (1750 rpm) under various oxygen and phosphorus concentrations and at different dilution rates. An in vivo Km for the oxygen-utilizing enzymes was found to be about 10% oxygen in the atmosphere, indicating the cultures would not be oxygen limited when growing in ordinary air. The respiratory index (R.I.= mmoles CO_2 produced / mg cells grown) leveled off gradually to a value of 0.05 below 15% oxygen and rose sharply above 15% oxygen in the gas phase, indicating a less efficient biomass production at higher oxygen concentrations. The amount of phosphorus required by the cells remained constant from 5 to 20% oxygen, suggesting that the higher rate of respiration was not coupled to ATP production. About 18 μg phosphorus were consumed by one mg dry weight organisms for cultures growing at oxygen concentrations in the gas phase below 5% and about 27 μg phosphorus per mg dry weight organisms for culures growing at oxygen concentrations from 5 to 20%. The growth rate of the culture was observed to play a role in the poly-β-hydroxybutyrate (PHB) accumulation in both phosphorus-limited and non-limited cultures. An accumulation of PHB up to 22% of dry weight was observed in phosphorus-limited cultures. The accumulation of PHB at the low dilution rate was about 50 fold greater than that at the high dilution rate. Electron micrographs also revealed the massive PHB accumulation at low dilution rates. Respiratory protection was shown to be present even in phosphorus-limited cultures by the higher R.I.'s under phosphorus-limited conditions. At the very low phosphorus concentration (0.07 mM) the rate of CO_2 evolution increased about 2 fold from D = 0.05 to D = 0.15 h^{-1}. At higher phosph

about 3 fold from D = 0.35 to D = 0.15 h^{-1}. At higher phosphorus concentrations the rate of CO_2 evolution remained unchanged for all dilution rates. Electron micrographs showed a larger amount of intracellular membrane vesicles at lower phosphorus concentrations. The function of the vesicles is thought to be to increase the cell surface and thus respiration. Mesosomes were thought to play a role in the increase in the amount of vesicles. Negative staining methods confirmed the membranous origin of the vesicles and revealed knob-like structures on the inside of the cell membrane.

INTRODUCTION

Introduction

The extremely high rate of respiration and the N_2-fixing ability of Azotobacter chroococcum have attracted many authors to investigate the growth and physiology of this bacterium. Dalton and Postgate (1969 a,b) were among the first to carry out studies on A. chroococcum in great detail. They found that A. chroococcum grew better at lower oxygen tensions. Carbon- and phosphate- limited cultures were more sensitive to oxygen inhibition than non-limited cultures. However, they found that carbon-limited cultures utilizing NH_4^+ seemed not to be so oxygen sensitive. Respiratory protection and conformational protection of the nitrogenase were proposed. They suggested that the carbon-limited culture did not get enough substrate to carry out respiratory protection and that the phosphate-limited culture did not have respiratory protection because the ATP/ADP ratio was affected by the lack of phosphate.

Lees and Postgate (1973) studied the behaviour of oxygen- and phosphate- limited A. chroococcum in continuous cultures. They reported that the inverse relation between biomass and dilution rate could be explained by the accumulation of poly-β-hydroxybutyrate and polysaccharide at lower dilution rates. They also confirmed the oxygen sensitivity of phosphate-limited nitrogen-fixing cultures. However, they discovered that the 'oxygen-poisoned' phosphate-limited cells were 100% viable when subsequently grown on medium containing NH_4^+. This, they thought, provided evidence for the idea that respiration protects the nitrogenase in nitrogen-fixing organisms.

Although the effect of oxygen on A. chroococcum has been extensively studied, in none of these studies were oxygen limitation and oxygen

sufficiency quantitatively defined. This inadequacy might be due to the complexity in measuring the degree of aeration in cultures (Brown, 1970). For instance, any change in the composition of the medium would change the solution rate of oxygen and so would the rate of stirring the culture. Therefore, in some cases, even though enough oxygen is supplied to the culture, the culture could still be oxygen limited if it is not stirred enough. Hine & Lees (1976) overcame this problem by growing *A. chroococcum* in continuous culture under vortex stirring (1750rpm). With this vortex stirring the transfer of oxygen and other gases to the cells in the culture is efficient, uniform and maximized. This is presumably due to an increase in the rate of gaseous diffusion to the cells. This gives more accurate measurements of the degree of oxygen limitation because the dissolved oxygen level measured would be very close to that of the cell surface. Using this intense rate of aeration Hine & Lees were able to determine oxygen limitation, oxygen sufficiency, and the transition between the two in terms of the respiratory index (R.I.) values (CO_2 evolved per weight of organisms produced). They showed that the amount of CO_2 evolved increased with the oxygen level and that above the R.I. value of 0.1 the respiratory protection came into function. With this stirring rate, they got a growth rate double that of Dalton & Postgate.

The aim of this investigation is to use this vortex stirring method to study the effect of phosphorus-limitation on *A. chroococcum* in continuous cultures under conditions of a regulated supply of oxygen by means of the vortex stirring.

HISTORICAL

Historical

Cellular nitrogen fixation is by far the most widespread process of fixing N_2 to NH_3. Molecular nitrogen is an inert gas in the atmosphere. The inertness is due to the stable triple bond in the nitrogen molecule. The outer shell of electrons of each atom in a nitrogen molecule has a neon electronic configuration, (8 electrons).

Nitrogen fixation is a part of the nitrogen cycle in nature and is quite often the limiting factor for the biological productivity in many areas, especially in agricultural land (Postgate,1978). The fixation has been known since 1888 when Hellriegel and Wilfarth proved its existence in legumes (Burns & Hardy,1975). However, active research into the process of nitrogen fixation started only about fifteen years ago when the acetylene reduction method coupled to gas chromatography was introduced to assay the nitrogenase activities (Dilworth ,1966; Koch & Evans, 1966; Hardy & Knight, 1967).

Biological nitrogen fixation is carried out exclusively by procaryotes and is found in the blue-green bacteria, the photosynthetic bacteria, the strict anaerobes and the obligate and facultative aerobes. A. chroococcum, the bacterium used throughout this investigation, is a strict aerobe, a heterotrophic nitrogen-fixer, found commonly in soil and water. It is rod shaped, about 2 x 5 μm, frequently in pairs, motile with peritrichous flagella and forms round cysts under adverse conditions (Bergey's Manual,1974).

Usually, organisms that fix N_2 are divided into two types, namely, symbiotic N_2-fixers and free-living N_2-fixers. The 'symbiotic N_2-fixers' refer to organisms that fix nitrogen only in association with their hosts,

mainly plants. Some examples of symbiotic N_2-fixers are: *Rhizobium* spp. with legumes, *Klebsiella pneumoniae* with tropical plants, *Spirillum lipoferum* with tropical grasses, and *Frankia* spp. with alder. Recently, some *Rhizobium* spp. have been found to fix nitrogen without the hosts (Keister,1975; Kurz & LaRue,1975; McComb *et al.*,1975; Pagan *et al.*,1975).

The study of nitrogen fixation was done on whole cells until the successful isolation of nitrogenase, the nitrogen-fixing enzyme complex, by Carnahan *et al.* (1960). The cell-free nitrogenase isolated was oxygen- and cold-labile. Since then nitrogenase has been isolated from about 20 microorganisms and purified from about 4 (Eady & Postgate,1974; Chatt *et al.*,1978). The amino-acid composition of the nitrogenases in *Azotobacter*, *Klebsiella* and *Clostridium* were compared (Chen *et al.*,1973) and that in *Clostridium pasteurianum* was partly sequenced (Tanaka *et al.*, 1976). The enzyme consists of two non-heme, iron-sulfur proteins. They are called MoFe protein (also called molybdoferredoxin or protein I) and Fe protein (also called azoferredoxin or protein II) and present in the ratio of 2:1 (MoFe protein:Fe protein) in the enzyme. The overall composition of these proteins is shown in Table 1. Although the exact molecular composition is specific to each species, the basic components and most of the amino-acids constituents are very similar. Components from some organisms have been shown to cross-react to give functional enzymes. For example, Fe proteins from *A. chroococcum* give functional nitrogenases when mixed with the MoFe protein of *Klebsiella pneumoniae* (Eady & Postgate,1974). Electron micrographs show that the MoFe protein consists of four doughnut-shaped units packed together and that the Fe protein is egg-shaped (Burns *et al.*,

Table 1 General properties of the components of the nitrogenase (Chatt *et al.*,1978)

Properties	MoFe protein	Fe protein
Molecular weight	~220,000[a]	55,000-70,000[a]
Number of subunits	2x2[b]	2
Iron atoms	20-32[a]	4
Molybdenum atom	2	0
Acid labile sulfide	~20-32[a]	4

[a] exact amount depends on the source

[b] two of each of two subunit types

1974). Both MoFe and Fe proteins are required for fixing nitrogen. Neither protein can fix nitrogen on its own.

Nitrogenase can react with many substrates. These include H_3O^+, and triple-bonded substrates such as N_2, N_2O, RCN, and RCCH, where R is either a hydrogen atom or an alkyl chain. For nitrogen fixation to occur in cellular extracts an external electron donor such as sodium dithionite, or potassium borohydride, a cation such as Mg^{++} and an ATP generating system such as the creatine-phosphate/creatine phosphokinase system must be present (Bulen et al.,1965). Studies show that the complex of Mg and ATP binds to the Fe protein and the MoFe protein is reduced by the Fe protein with the involvement of Mg·ATP (Mortenson et al.,1973; Orme-Johnson et al.,1972; Shah et al., 1973; Smith et al., 1973). It was proposed that the Mo-Fe-S in the enzyme is the site for the reduction of the nitrogen molecule. The reduced Fe protein transfers its electron to the MoFe protein and then to the nitrogen molecule bound to the active site. A series of electron transfers takes place and protons are also transferred from water to the nitrogen molecule. The result is ammonia, Mg·ADP and inorganic phosphate (P_i) are produced with the nitrogenase going back to its original oxidation state. The ammonia formed is then released from the enzyme. Diazene and hydrazine are believed to be the intermediates of the reduction and are bound to the transition metal all the time at the active site. A tentative scheme of the mechanism of nitrogen fixation is shown in figure 1. There are still uncertainties in the mechanism of nitrogen fixation. The evidence for molybdenum to be the site of attachment of the nitrogen molecule is very indirect (Smith,

Fe protein (oxidized)

↓ $Na_2S_2O_4$ or reduced forms of flavodoxins, ferredoxins or Viologen

Fe^- protein — MgATP; MoFe protein — N_2

↓

Fe^- protein, Mg·ATP, MoFe protein, N_2

↓ protein–protein electron transfer

Fe protein, Mg $\{^{ATP}_{ADP}\}$, $MoFe^-$ protein, N_2

↓ 5 more of the above electron-transfer steps + $6H^+$

Fe protein (oxidized) + MgADP

+ P_i + MoFe protein + $2NH_3$

Figure 1 Simplified scheme of nitrogenase action. The scheme shows the steps believed to be involved in nitogenase action; but the sequence shown is not firmly established (Chatt et al.,1978).

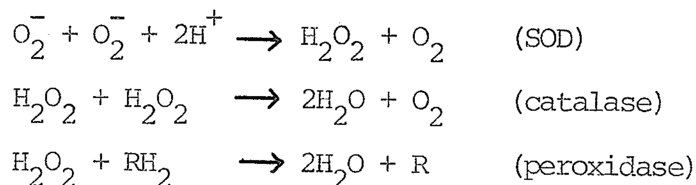
1977, cited in Chatt et al.,1978). Whether the iron-sulfur clusters in the enzyme are electron donors only or also interact with the nitrogen molecule is not certain. Recently, the long hypothesized intermediate hydrazine has been isolated when the enzyme is destroyed by acid or alkali during the fixation process (Thorneley et al.,1978). However, the other hypothesized intermediate diazene has not been detected yet.

A side issue of the nitrogen fixing reaction is the production of hydrogen. Nitrogenase is a powerful reducing agent. In the presence of nitrogen, the nitrogen is reduced. However, when nitrogen is absent, H^+ in water is reduced to hydrogen. The hydrogen production is also ATP dependent and is present both in cell-free extracts (Burns & Hardy, 1975; Zumft & Mortenson,1975) and *in vivo* (Hamilton et al.,1964; Walker & Yates,1979). Since CO does not inhibit hydrogen production but inhibits nitrogen fixation it is believed that the catalytic site of the proton reduction is not the same as that of nitrogen reduction. Competition seems to exist between the H^+ and the N_2 for the reduction because in the presence of N_2, H_2 evolution is much reduced.

The source of electrons and ATP for the nitrogenase in *Clostridium pasteurianum* is the phosphoclastic breakdown of pyruvate (Evans & Phillips,1975). In strict aerobes the ATP is believed to be provided by oxidative phosphorylation (Yates & Jones, 1974). The path for the electron transfer from NADPH is not clear yet. In *A. vinelandii* (Benemann et al.,1971) and soybean bacteroids (Evans & Phillips,1975) the proposed sequence is: NADPH $\longrightarrow$ ferredoxin $\longrightarrow$ x $\longrightarrow$ flavodoxin $\longrightarrow$ nitrogenase. The NADPH could be generated from a number of $NADP^+$-linked dehydrogenases. The component x has not been fully identified.

Although nitrogen fixation is an exothermic reaction, a large amount of ATP is required for the process. Estimates of ATP required for nitrogen fixation *in vivo* varies from 4-5 ATP/N_2 in *A. chroococcum* (Dalton & Postgate, 1969b) to 29 ATP/N_2 in *Klebsiella pneumoniae* (Hill, 1976), while that for cell-free extract is about 15 ATP/N_2 (Silver, 1967). The exact role of ATP in nitrogenase is not known. During the fixation ATP is hydrolysed to ADP and P_i (Hardy & Knight,1966; Kennedy *et al.*, 1968). It is suggested that the binding of the ATP to the nitrogenase complex may enhance the catalytic activity of the transition metal in the complex. (Yates,1972; Zumft *et al.*,1972,1973).

Nitrogenase is extremely sensitive to oxygen, especially the Fe protein which has a half life of less than 1 minute on exposure to air (Hardy & Havelka,1975). The inactivation of the cell-free nitrogenase by oxygen is irreversible. The toxicity of oxygen has been well documented (Fridovich,1974,1975a,b,1978). The metabolism of oxygen in the cell can easily produce the highly reactive superoxide anion, O_2^-. This anion has been shown to inactivate virus, induce lipid peroxidation, damage membranes, and kill cells (Fridovich,1978). The mechanism of toxicity of the O_2^- is probably through the formation of the extremely powerful oxidant, the hydroxyl radical (OH•), when H_2O_2 is present: $H_2O_2 + O_2^- \longrightarrow OH^- + OH\cdot + O_2$. Since O_2^- and H_2O_2 are regularly produced in cells, the cells have developed enzymes to get rid of these harmful products as soon as they are formed, to prevent them from reacting with one another. The enzyme that removes O_2^- is superoxide dismutase (SOD) and the enzymes that remove H_2O_2 are catalase and peroxidase. The reactions are as follows:



$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad \text{(SOD)}$$

$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2 \qquad \text{(catalase)}$$

$$H_2O_2 + RH_2 \longrightarrow 2H_2O + R \qquad \text{(peroxidase)}$$

It is not known how nitrogenase is inactivated by oxygen. It may be due to the effect of O_2^-. However, there is much evidence that oxygen affects the physiology of the nitrogen-fixing microorganisms and that the microorganisms have developed mechanisms to protect their nitrogenases from oxygen damage. Nitrogen-fixing facultative aerobes such as Klebsiella or Bacillus and nitrogen-fixing photosynthetic procaryotes fix nitrogen only under anaerobic conditions. Some blue-green bacteria carry out nitrogen fixation in heterocysts only where the oxygen tension is low (Stewart,1978). In legumes, the plant and the bacteroids co-operate to produce leghaemoglobin to maintain a suitable oxygen-tension 10nM in the bacteroids for them to fix nitrogen. The strict aerobes face a bigger problem. They have to live in the presence of oxygen and yet they have to keep the nitrogenase in an anaerobic environment for the nitrogenase to function. The results of many investigations show that the aerobes have developed many extraordinary protective mechanisms to cope with excess oxygen supply. The following is an account of these possible protective mechanisms found in Azotobacter species.

A. chroococcum has been shown to contain SOD and catalase and the amount of these two enzymes increases when the oxygen level supplied to the cells increases (Buchanan,1977). So these may indicate that the two enzymes are used in protecting nitrogenase from oxygen damage.

In addition to the production of SOD and catalase azotobacters

when given sufficient carbon substrate can significantly increase their respiration rate in response to the increase in oxygen supply. This augmented respiration was interpreted as respiratory protection (Dalton & Postgate,1969a,b). It was suggested that the cell increases its respiration to burn away excess oxygen so that an intracellular anaerobic condition is maintained for nitrogen fixation.

One consequence of this augmented rate of respiration is the production of excess ATP because repiration usually produces ATP. This excess ATP would upset the steady intracellular ATP:ADP ratio, which if too high would inhibit nitrogenase activities (Yates,1970). However, it was found that the respiratory chain in Azotobacter is branched and one of the branches is uncoupled with respect to ATP formation (Jones & Redfearn,1967) (figure 2). At high respiration rate electrons could be transported via the less coupled pathway, i.e., from NADH dehydrogenase to cytochrome a_2. This would enable the cell to respire at a higher rate and not to produce excess ATP. On the other hand when the oxygen level is low a pathway with high phosphorylating efficiency predominates, producing enough ATP for cellular functions and nitrogen fixation. Bergerson and Turner (1975) also reported the presence of branched electron transport system in legume bacteroid system.

Dalton & Postgate (1969a) also suggested the presence of conformational protection in Azotobacter. This means that the nitrogenase in vivo can switch from a functional and oxygen sensitive form at low oxygen tension to a non-functional, oxygen insensitive form at a sudden increase of oxygen tension and vice versa. However, much more experimental evidence is needed to confirm this idea.

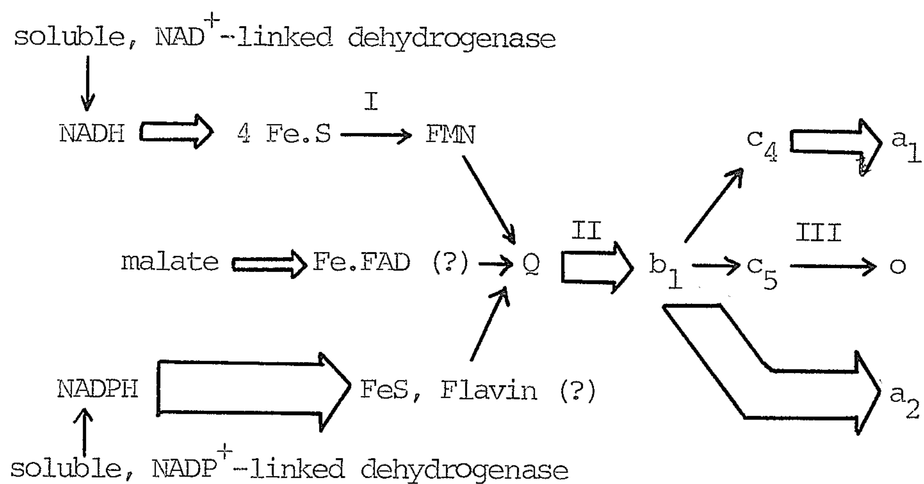


Figure 2 Cytochrome-linked electron transport pathways in _A. vinelandii_. I, II, III represent the three phosphorylation sites. Thickness of the arrows represents the effective electron flux through a particular pathway. (Jones & Redfearn,1967, cited in Dalton,1979)

Another protective mechanism which may be present in *Azotobacter* is the production of a lot of exopolysaccharide. There is evidence that the bacterial gum would slow down the rate of diffusion of oxygen (Hill *et al.*,1972; Hine & Lees,1976). This could protect the nitrogenase in the cell temporarily from sudden changes in oxygen tension in the environment.

The cell membrane of *Azotobacter* may also play a role in protecting the nitrogenase from oxygen inactivation. For instance, nitrogenase extracted from *A. vinelandii* is not oxygen-labile. The extract is particulate and contains membrane fragments (Bulen *et al.*,1964; Hardy & Knight,1966). Also, an extensive intracellular membrane network or membrane invaginations has been repeatedly reported in *Azotobacter* under various growth conditions. The possible function of the membrane in protecting the nitrogenase *in vivo* will be detailed later.

The ability of *Azotobacter* to accumulate large amounts of poly-β-hydroxybutyrate (PHB) is probably another protective mechanism against oxygen inactivation too. This will be discussed in more detail.

So, it seems that the *Azotobacter* has evolved quite a number of mechanisms, namely, SOD and catalase production, high respiratory rate, conformational change of the nitrogenase, a branched oxidative electron-transport system, exopolysaccharide production, extensive intracellular membrane network and PHB accumulation, to protect the nitrogenase from oxygen inactivation.

PHB accumulation

Many bacteria are able to accumulate PHB . Accumulation up to 70% of the cell dry weight has been reported in *A. beijerinckii* (Stockdale

et al.,1968). PHB was discovered by M.Lemoigne in 1927 and has a molecular weight ranging from 1,000 to 256,000 (Doudoroff,1966), depending on the growth condition the cell is in. It is a polymer of β-hydroxybutyrate and is usually accumulated into protein-coated granules (Wang & Lundgren, 1969). The compostion of a PHB granule is 98% PHB, 2% protein and trace amounts of lipid and phosphorus (Doudoroff,1966).

PHB is considered to be an intracellular reserve of carbon and/or energy that is stored up when the carbon substrate in the environment is in excess and is used when the carbon substrate in the environment is exhausted (Senior *et al.*,1972). Its role in endogenous metabolism has been studied extensively (Dawes & Ribbons,1964). In *Azotobacter* PHB is probably present in small amounts all the time (Stockdale *et al.*,1968). However, massive accumulation can occur when the cells get into the stationary phase of growth in the presence of excess carbon substrate (Wyss *et al.*,1961; Senior *et al.*,1972; Sadoff,1975). PHB accumulation has always been observed to precede encystment in *Azotobacter*. However, PHB accumulation does not necessarily result in cyst formation. In batch cultures unfavorable conditions such as mineral deficiencies would result in cyst formation in *A. vinelandii* (Layne & Johnson,1964) and so would the addition of n-butanol (Wyss *et al.*,1961). Since PHB accumulation is also observed prior to the encystment it can be said that the above mentioned unfavorable conditions may actually first effect a PHB accumulation. In continuous cultures PHB accumulation was observed in *A. chroococcum* at low dilution rates in phosphate-limited, N_2-limited, NH_4^+-limited and oxygen-limited cultures (Dalton & Postgate,1969b; Lees & Postgate,1973).