Changes in Respiratory Activity during Encystment of *Azotobacter vinelandii*

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Vegetative cells of *Azotobacter vinelandii* were induced to encyst in the presence of β -hydroxybutyrate. Although modal cell volume fluctuated (2.7 to $4\ \mu\text{m}^3$) markedly during exponential growth, the final modal volume attained by mature cysts (1.13 to $1.3\ \mu\text{m}^3$) was relatively constant. Respiratory activity of encysting cultures fell rapidly 3 h after initiation of encystment. This was accompanied by decreased inhibition of respiration by KCN. NADH oxidase activity of membrane preparations fell from approximately 450 to $50\ \text{nmol O}_2\ \text{consumed min}^{-1}\ (\text{mg protein})^{-1}$ during differentiation and was also less sensitive to KCN. Difference spectra of cytochromes in membrane preparations revealed a number of changes. Amounts of cytochromes *b*, *c* and *a*₁ all fell during encystment; cytochrome *o* levels increased whilst cytochrome oxidase *d* was gradually lost from the membrane during the first 22 h of morphogenesis.

INTRODUCTION

Vegetative cells of *Azotobacter vinelandii* can be induced to encyst in the presence of β -hydroxybutyrate as the sole carbon source. The process occurs in approximately 65 h at $30\ ^\circ\text{C}$ (Socolofsky & Wyss, 1961; Lin & Sadoff, 1968). During this period of differentiation motile cells lose their flagella, round up and secrete membrane-like vesicles into the capsular material to form a dense shingled structure called the exine. This encloses a less dense intine layer and the cyst central body. The cysts are spherical, metabolically dormant, refractile and more resistant to deleterious physical and chemical changes than are the cells (Sadoff, 1975). Enzyme activity, structural development and lipid composition have all been investigated during encystment (Hitchins & Sadoff, 1970, 1973; Reusch & Sadoff, 1979, 1981). Changes in lipid composition have been particularly well characterized. A major feature is that the phospholipids of the cell membrane are replaced by a family of 5-*n*-alkyl resorcinols and 6-*n*-alkyl pyrones during encystment. These novel lipids are synthesized from 8 h after the induction of encystment and it has been proposed that they may form a unique membrane matrix which contributes to the physiology and properties of the cyst (Reusch & Sadoff, 1983).

Recently, we have shown that there are significant differences between vegetative cells and cysts with respect to respiratory enzyme activities, cytochrome content and extent of inhibition of respiration by KCN. The respiratory activity of cysts is some 10-fold lower and cytochrome oxidase *d* is apparently absent. Respiration of cysts is also much less sensitive to KCN (Field & Edwards, 1983). In this paper we examine the time course of changes in respiratory activity during encystment. Complex modulation of respiration, sensitivity to KCN and cytochrome content are revealed. These changes are discussed in relation to other known temporal events that occur after induction of encystment.

METHODS

Growth and maintenance of the bacteria. *Azotobacter vinelandii* (NCIB 8789) was grown in a N-free medium that contained, per litre: Na_2HPO_4 , 0.82 g; KH_2PO_4 , 0.86 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; CaCl_2 , 50 mg; Na_2MoO_4 , 5 mg; nitritoltriacetic acid, 1 mg; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 11 mg; citric acid, 10 mg; mannitol, 15 g. Cultures were maintained on

plates of Lab M (Pendleton, Salford, UK) agar made up to 1% (w/v) with the N-free medium. Batch cultures were grown in 400 ml growth medium in 2 litre flasks with shaking at 30 °C. Large-scale cultivation was in 4–6 litre amounts using a Churchill fermenter (L.H. Engineering, Stoke Poges, Bucks) at a stirring rate of 200 r.p.m. and aeration of 1 litre air min⁻¹ (1 culture)⁻¹. Growth was followed either by measuring the OD₅₅₀ with a CE 272 spectrophotometer (Cecil Instruments Cambridge), or by counting cell numbers of culture samples appropriately diluted in Isoton 11 with a ZBI Coulter counter (Coulter Electronics, Harpenden, Herts). Cell volumes were measured with a C-1000 channelyzer linked to the Coulter counter after calibration of the latter with latex beads of 1.15 µm diameter.

Induction of encystment. Exponentially-growing vegetative cells (OD₅₅₀ 0.6–0.8) were pelleted by centrifugation at 17000g for 20 min and washed once with sterile 20 mM-potassium phosphate buffer, pH 7.0. The cells were finally resuspended in a volume equal to that of the original culture using growth medium that contained 0.2% (w/v) β-hydroxybutyrate as the sole carbon source. Almost complete encystment occurred when these cells were incubated for 65 h with shaking at 30 °C.

Preparation of membranes. Cell or cyst suspensions were disrupted using a modification of the lytic system of Repaske (1958). Vegetative cells or cysts were suspended in 33 mM-Tris/HCl buffer, pH 8, which contained EDTA (133 µg ml⁻¹) and lysozyme (33 µg ml⁻¹). After incubation for 10 min with occasional stirring the suspension was sonicated at a setting of 12 µm for 3 × 1 min periods with an MSE ultrasonic disintegrator. Unbroken cells and debris were removed by centrifugation at 17000g for 20 min. Membranes were sedimented from the resultant supernatant by centrifugation at 180000g for 1 h at 4 °C. The pellets were resuspended in 20 mM-phosphate buffer, pH 7.0, and these membrane preparations used immediately.

Measurements of oxygen uptake. Oxygen uptake rates by cell suspensions during encystment were measured at 30 °C with a Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, UK) linked to an MSE Vitatron chart recorder.

Cytochrome content. Changes in amounts of cytochromes during encystment were detected from difference spectra of membrane preparations in 2 × 1 ml matched glass cuvettes of 0.5 cm path length. Spectra were plotted at room temperature on an SP1800 spectrophotometer. Reduction was by the addition of a few grains of sodium dithionite to one cuvette and oxidation by vigorously shaking the contents of the other cuvette. For CO-difference spectra, both cuvettes were reduced, and CO bubbled through the contents of one cuvette for 5 min. The reduced-plus-CO minus reduced difference spectrum was then recorded. Cytochromes were quantified using the following mM absorption coefficients (wavelength pairs are in parentheses): *a*₁, 4.6 (603–575); *b*, 17.5 (560–570); *c*₄ + *c*₅, 17.5 (553–538); *d*, 8.5 (630–655); *o*, 170 (418–390).

Determination of extracellular polysaccharide and dry weight. Exopolysaccharide production during exponential growth was measured by the alcohol precipitation method of Jarman *et al.* (1978). Samples (40 ml) were removed at intervals from a batch culture (4 l) and 5 M-NaCl and 0.5 M-EDTA (0.8 ml of each) added. After centrifugation at 24000g for 40 min at 4 °C the supernatant was removed and mixed vigorously with 75 ml propan-2-ol for 30 s. After standing for 10 min at room temperature the resultant precipitate was filtered on to a Whatman GF/A filter which had been dried overnight at 110 °C and weighed prior to use. The precipitate was washed on the filter with 100 ml propan-2-ol/H₂O (3:1, by vol.), dried and weighed to give the weight of extracellular polysaccharide in 40 ml culture. The cell pellet from the first centrifugation step was washed in water, pelleted at 24000g for 20 min, resuspended in distilled water (2 ml) and transferred to a dried preweighed glass vial. The suspension was dried overnight at 110 °C and the vial weighed to yield the dry weight of cells from 40 ml culture.

RESULTS

Changes in modal cell volume during vegetative growth and encystment

Experiments to investigate changes in volume during differentiation of cells into cysts revealed great variability in the modal cell volume of exponentially-growing vegetative cells. The results of a typical experiment are shown in Fig. 1. After inoculation of cells from the late exponential phase into growth medium, modal cell volume rose from 2.3 µm³ at 1 h to 3.2 µm³ at 4 h and then gradually fell to 2.4 µm³ at 8 h. During this time OD₅₅₀, cell numbers and dry weight all increased at similar rates (doubling time 180 min) indicating that balanced growth (Campbell, 1957) was occurring. The fluctuation in volume must have been due to another factor(s). One possibility is that the extracellular polysaccharide alginic acid produced by *A. vinelandii* (Gorin & Spencer, 1966) affects the measurements of cell volume. However, during vegetative growth this polymer was produced continuously but at a lower rate than the increase in cell numbers and therefore did not contribute to the changes in cell volume (Fig. 1). A similar result was obtained for cells grown in the presence of NH₄Cl, in which no cell volume changes can be detected during exponential growth.

Fig. 1

Fig. 2

Fig. 1. Changes in modal cell volume and exopolysaccharide production during exponential growth of *A. vinelandii*. Exponentially-growing cells were inoculated into growth medium that contained mannitol as the carbon source, and samples were removed from the culture at time intervals for the determination of cell numbers (●), OD_{550} (○), exopolysaccharide production (▲), cell dry weight (△) and modal cell volume (■). Results are from a typical experiment.

Fig. 2. Changes in modal cell volume and cell numbers after the induction of encystment of vegetative cells of *A. vinelandii*. Washed vegetative cells were inoculated into growth medium (at time 0) that contained β -hydroxybutyrate as the sole carbon source. Cell numbers (●), OD_{550} (○) and modal cell volumes (■) were measured during the subsequent period of differentiation. Results are from a typical experiment.

Table 1. Cell division and decrease in volume of *A. vinelandii* during encystment

Experimental details were as described for Fig. 2. Results are from a typical experiment.

$10^{-8} \times \text{Cell numbers ml}^{-1}$			Modal cell volume (μm^3)		
At induction	After encystment	Factor of increase	At induction	After encystment	Factor of decrease
2.36	3.85	1.63	2.70	1.28	2.11
1.63	3.75	2.30	3.30	1.28	2.58
1.52	6.15	4.05	3.98	1.13	3.52

The relationship between the initial cell volume and final cyst volume was investigated by inducing different batch cultures, each of which contained cells of a known modal volume, to encyst (Table 1). The final volume of mature cysts (1.13 – $1.28 \mu\text{m}^3$) was similar irrespective of the initial volume of encysting cells. Exposure of cells to β -hydroxybutyrate resulted in cell division sufficient to attain the volume characteristic of cysts. Therefore some cells divided more than once before they developed into mature cysts and the number of divisions was related to the modal volume of the induced vegetative cells.

Changes in cell numbers, OD_{550} and modal volume during encystment are shown in Fig. 2. In this experiment cell numbers rose from $2.6 \times 10^8 \text{ cells ml}^{-1}$ to $6 \times 10^8 \text{ cells ml}^{-1}$ after 5 h. The OD_{550} rose continuously (doubling time approximately 8 h) from 0.5 to 0.9 after 8 h and gradually increased to 1.3 after 60 h, presumably reflecting the increased refractility of cysts.

Fig. 3. Respiratory activity and changes in cyanide sensitivity during encystment. Samples (2.4 ml) were removed at intervals from an encysting culture of *A. vinelandii* and transferred directly to the chamber of an oxygen electrode. The oxygen uptake rate (■) was determined for each and KCN at a concentration (0.1 mM) sufficient to inhibit the respiration of vegetative cells by 50% was then added. A $t_{1/2}$ value (□) was calculated by determining the time taken for this concentration of inhibitor to reduce the respiration rate to half of its initial value. Results are from a typical experiment.

Modal volume decreased rapidly from $3.33 \mu\text{m}^3$ to $1.13 \mu\text{m}^3$ after 7 h and remained at this level for 60 h, by which time mature cysts had been formed. The rapid drop in modal volume occurred concomitantly with the steepest rise in cell numbers.

Oxygen uptake and the effects of cyanide during encystment

We have shown previously that the respiratory activity of cysts is much lower than that of cells and is much more resistant to KCN (Field & Edwards, 1983). The time course of modulation of terminal respiration was therefore investigated during encystment. Cyanide, at a concentration (0.1 mM) sufficient to inhibit the respiration of vegetative cells by 50% was added to samples removed at different stages of encystment. The time taken to inhibit respiration by 50% increased during differentiation. In order to compare the effects of the inhibitor at different times of encystment a $t_{1/2}$ value (time taken to reduce oxygen uptake to 50% of the untreated value) for inhibition of respiration by 0.1 mM-KCN was calculated for each sample. Both respiration rate and $t_{1/2}$ remained constant up to 3 h after induction of encystment (Fig. 3). Thereafter, oxygen uptake rates fell rapidly from approximately 12 to $0.75 \text{ nmol O}_2 \text{ consumed min}^{-1} (10^8 \text{ cells})^{-1}$ after 8 h. The uptake rate remained at the latter value for the remainder of the differentiation period and is characteristic of mature cysts. The $t_{1/2}$ value rose from approximately 1 min at 3 h post-induction to 8 min by 8 h, indicating the gradual acquisition of resistance to cyanide during the early stages of morphogenesis. The final $t_{1/2}$ value for mature cysts was 20 min.

Effects of cyanide on NADH oxidation by membrane preparations

The increasing resistance of encysting cells to KCN could reflect decreasing permeability to the inhibitor due to reorganization of surface layers (see Sadoff, 1975). Therefore the activity of NADH oxidase in membrane preparations was measured in the presence and absence of KCN at different times of encystment. NADH oxidase activity ($\text{nmol O}_2 \text{ min}^{-1} \text{ mg protein}^{-1}$) remained constant at 450 up to 4 h after induction. Thereafter it fell rapidly to reach a value of 75 at 24 h and 50 at 60 h (Fig. 4). The latter value is characteristic of cyst membranes. The $t_{1/2}$ value for inhibition of NADH oxidase by $200 \mu\text{M}$ -KCN (sufficient to inhibit oxidase activity in

Fig. 4

Fig. 5

Fig. 4. Respiratory activity and changes in cyanide sensitivity during oxidation of NADH by respiratory membranes prepared from samples removed at known times from an encysting culture. Oxygen uptake rates (■) by membrane preparations were measured in the presence of 50 mM-NADH. Cyanide (200 μ M), sufficient to inhibit NADH oxidation in membrane preparations from vegetative cells by 50%, was then added. The $t_{1/2}$ value (□) was then calculated as in Fig. 4. Results are from a typical experiment.

Fig. 5. Cytochrome levels during encystment. Culture samples were removed at time intervals during encystment and used to prepare membrane fractions. Difference spectra were plotted for each membrane sample and used to calculate the amount of total cytochrome *c* (●); cytochrome *b* (○); cytochrome *a*₁ (▼); cytochrome *d* (▽) and cytochrome *o* (■). The cytochrome concentrations in mature cyst membranes are shown on the right (results from Field & Edwards, 1983; means of five determinations).

vegetative cell membranes by 50%) rose from 30 s at 8 h to approximately 76 s at 60 h. This showed that the changes in sensitivity to KCN observed during encystment reflected membrane composition and activity rather than a permeability effect.

Changes in cytochrome content during encystment

Changes in respiratory activity during encystment were further characterized by measuring the concentrations of cytochromes from difference spectra of suspensions of cytoplasmic membranes. Cytochromes *b* and *a*₁ decreased in amount during the first 20 h of encystment to reach approximately 80% and 40% respectively of the contents of vegetative cells. Thereafter the concentrations of these cytochromes remained constant during differentiation. Total cytochrome *c* showed a slight increase in amount during the first 4 h of encystment but then fell to reach 72% of the initial value by 20 h. Cytochrome *d* decreased from 0.58 nmol mg protein⁻¹ to undetectable levels by 22 h. In contrast, the concentration of cytochrome oxidase *o* rose from 0.15 nmol mg protein⁻¹ to 0.34 nmol mg protein⁻¹ (Fig. 5). Mean values (five determinations) taken from Field & Edwards (1983) of the concentration of the cytochromes in membranes prepared from mature cysts are also shown in Fig. 5. These indicate that no further changes occur in cytochrome content after the initial 20–22 h of encystment.

DISCUSSION

The changes in modal volume that occur during exponential growth of vegetative cells of *A. vinelandii* are not due to the production of extracellular polysaccharide. They most likely reflect growth under N₂-fixing conditions. Post *et al.* (1982) found that N₂-fixing cells of *A. vinelandii*

Fig. 6. Comparison of the time course of modulation of respiratory activity (this paper) with changes previously reported for other parameters during encystment. The references are as follows; 1, Hitchins & Sadoff (1973); 2, Reusch & Sadoff (1981); 3, results from this work.

growing in continuous culture increased in volume in response to raised oxygen tensions. They proposed that this was due to an increase in the amount of intracytoplasmic membrane. Such a response could explain the results reported here. Table 1 shows that highest cell volumes were found at low culture densities and hence greater oxygen tensions. This view is supported by the fact that cells growing in the presence of NH_4Cl as a nitrogen source (i.e. under non-fixing conditions) do not fluctuate in volume during exponential growth (unpublished observations).

The characteristic variations in modal cell volume have allowed us to examine the relationship between the volume of cells at induction and the final volume of mature cysts. Addition of β -hydroxybutyrate triggers off reductive cell division which results in successively smaller progeny until at 8 h the characteristic cyst volume of approximately $1.2 \mu\text{m}^3$ is attained. Some cells divide more than once during this process, the number of divisions being related to the volume of cells at the commencement of encystment.

The timing of the various phases of respiratory activity reported here can be compared with previously published results for a number of other events which occur during encystment. Key metabolic enzymes fluctuate in activity and rise to peak values at characteristic times during encystment. For example β -hydroxybutyrate dehydrogenase, aldolase, isocitrate lyase, fructose-1,6-bisphosphatase and malate synthase all have two maxima of activity during encystment (Fig. 6). Within 4 h of induction nitrogenase activity becomes undetectable, DNA synthesis is switched off and phospholipid formation levels off. Cell division continues up to 8 h.

Respiratory activity remains constant up to 3 h but thereafter declines rapidly. Presumably a high rate of oxygen consumption for respiratory protection of nitrogenase (Jones *et al.*, 1973) is unnecessary because the activity of this enzyme has disappeared after approximately 2 h. The rapid fall in respiratory activity is accompanied by increased resistance to KCN which suggests that changes in the terminal oxidases of the bacterium are occurring. This proposal is supported by the fact that whole cysts (unlike cells) yield biphasic curves for the inhibition of respiration by KCN and that I_{50} values for the inhibition of oxidation of NADH and NADPH by KCN in cyst membrane preparations are approximately 10-fold higher than those recorded for membranes from vegetative cells (Field & Edwards, 1983). From previous work on electron transport in *A. vinelandii* one might predict that increased resistance to cyanide could result from increased synthesis of cytochrome oxidase *d* (Haddock & Jones, 1977; Jurtshuk & Yang, 1980). However, cytochrome *d* is in fact lost during differentiation (0–22 h). The concentrations of cytochromes *b*, *c* and a_1 also fall during this time, whereas the concentration of cytochrome oxidase *o* rises. The possibility that increasing resistance to cyanide reflects changes in the permeability of the membrane to the inhibitor due to modulation of membrane lipid content is unlikely because the NADH oxidase activity of membrane preparations also falls and becomes less sensitive to inhibition by KCN. Taken together, these results can be interpreted in one of two ways. Firstly, modulation of respiratory activity and cyanide sensitivity is a consequence of changes in the terminal oxidases. Support for this comes from the knowledge that NADH oxidation proceeds principally *via* the *b*–*d* route (Hoffman *et al.*, 1979, 1980*a, b*). Thus, loss of cytochrome *d* might result in the gradual lowering of NADH oxidase activity. Increased sensitivity to cyanide is more difficult to explain. One possibility is the synthesis of a second type of cytochrome *o* oxidase during encystment. This is a strong possibility because amounts of cytochrome *o* increase significantly during encystment (Fig. 5). Two types of cytochrome *o* differing in their sensitivity to KCN have been detected in *A. vinelandii* (Erickson & Diehl, 1973; Jurtshuk & Yang, 1980). Therefore, increased resistance of cysts to cyanide may be due in part either to increased amounts of this cyanide-insensitive *o*-type cytochrome oxidase or to an auto-oxidizable type *b* cytochrome which has been proposed by Kauffman & Van Gelder (1974) to mediate a third cyanide insensitive branch of the respiratory chain.

Secondly, it could be hypothesized that changes in respiratory activity occur as a result of the synthesis of novel membrane lipids. Phospholipid synthesis declines and levels off during the first 4 h of encystment, and shortly after this time the synthesis of alkyl resorcinols AR1 and AR2 commences and continues up to approximately 36 h (AR1) and 48 h (AR2) post-induction. These lipids may alter the environment of respiratory proteins so drastically as to exclude cytochrome *d*, and change membrane structure and function sufficiently to dampen the activity of the respiratory chain. The novel lipids synthesized during the encystment of *A. vinelandii* would therefore be a major contributory factor to the metabolic dormancy of the cyst (Reusch & Sadoff, 1983). Further work will concentrate on characterizing the cyanide insensitivity of cysts and attempting to distinguish alterations in membrane structure from those of function and activity.

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