RESPIRATORY PROTECTION OF NITROGENASE IN *AZOTOBACTER VINELANDII*

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1. Introduction

Azotobacter spp. are obligately aerobic bacteria which are capable of reductively assimilating atmospheric nitrogen via a particulate, oxygen-sensitive nitrogenase [1, 2]. Studies with *A. chroococcum* under continuous culture [3] suggest that, in the presence of excess oxygen, the nitrogenase is protected by a dual mechanism consisting of:

i) *Conformational protection*, a reversible process, probably manifested via a rapid conformational change within the nitrogenase complex which causes the oxygen-sensitive sites to become inaccessible to oxygen and the nitrogenase to become temporarily inactive, and

ii) *Respiratory protection*, which removes the excess oxygen via enhanced respiration and causes reversal of the conformational protection process (see [4, 5]).

More recent studies on the complex respiratory system of batch-cultured *A. vinelandii* [6–8] clearly show that the ambient oxygen concentration during growth has profound effect upon the activity and composition of the respiratory chain of this organism. This present paper describes the changes which occur within the respiratory system of *A. vinelandii* following the sudden exposure of low aeration cultures to excess oxygen conditions and which, by lowering the energy conservation efficiency, appear to be responsible for the resultant phenomenon of enhanced respiration.

2. Materials and methods

Azotobacter vinelandii (NCIB 8660) was batch-cultured at 30° on N_2-mannitol medium [9] at low aeration and then exposed to extra-high aeration conditions (144 mmole oxygen·ℓ^{-1}·hr^{-1}) as described previously [7].

Whole cell cytochrome spectra were determined by reflectance spectrophotometry using a Shimadzu MPS 50L recording spectrophotometer and cytochrome concentrations (expressed as $\Delta A \cdot g$ dry wt^{-1}) were calculated using the following wavelength pairs, $\Delta A_{548-535}$ nm ($c_4 + c_5$), $\Delta A_{555-575}$ nm ($b_1 + o$) and $\Delta A_{627-612}$ nm (a_2); the technique was not sufficiently sensitive to allow the assay of cytochrome a_1. The intracellular ATP concentration was determined, following rapid sampling, by an essentially standard luciferin–liciferase method [10]. Whole cell respiratory activity (Q_{O_2}) was determined polarographically as described previously [7]. Phosphorylating repiratory membranes were prepared and assayed for oxygen uptake, $AT^{32}P$ synthesis and cytochrome content by previously described procedures [6, 7]. Protein was determined by the modified Biuret method [11].

3. Results

When batch cultures of *A. vinelandii*, grown under nitrogen-fixing conditions but with low aeration, were exposed to extra-high aeration conditions, an immediate cessation of growth and a rapid increase in whole cell respiratory activity (Q_{O_2}) were observed. The Q_{O_2} reached a maximum value after 3.5–4.0 hr exposure and this event was accompanied by the resumption of rapid, logarithmic growth. A large number of these

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Abbreviation:
m-Cl CCP, carbonylcyanide *m*-chlorphenyl hydrazone.

Table 1

The effect on *A. vinelandii* whole cell respiratory activity of changing the growth conditions from low to extra-high aeration.

Additions	Cell density at changeover (mg dry wt·ml^{-1})	Q_{O_2} 0 hr (μl O_2·hr^{-1}·mg dry wt^{-1})	Q_{O_2} 4 hr (μl O_2·hr^{-1}·mg dry wt^{-1})	ΔQ_{O_2}
Nil (Control)	0.106 ± 0.035	424 ± 132	909 ± 227	485
Chloramphenicol (200 μg/ml)	0.082 ± 0.008	470 ± 129	728 ± 256	258

Cell culture and aeration changeover conditions as described in the Materials and methods section. The values for Q_{O_2} and cell density quoted in the table refer to the average ± the standard deviation from 10 control experiments and 4 *plus*-chloramphenicol experiments.

Table 2

The effect on *A. vinelandii* whole cell cytochrome and ATP concentrations of changing the growth conditions from low to extra-high aeration.

Time after aeration changeover (hr)	Experiment A (Control - no additions) Cytochromes c_4+c_5 (ΔA·g dry wt^{-1})	Cytochromes b_1+o (ΔA·g dry wt^{-1})	Cytochromes a_2 (ΔA·g dry wt^{-1})	ATP (nmole·mg dry wt^{-1})	Experiment B (+ 200 μg·ℓ^{-1} chloramphenicol) Cytochromes c_4+c_5 (ΔA·g dry wt^{-1})	Cytochromes b_1+o (ΔA·g dry wt^{-1})	Cytochromes a_2 (ΔA·g dry wt^{-1})
0	6.43	4.55	0.84	5.60	10.40	7.34	1.48
1	6.17	4.35	0.97	4.30	11.20	7.81	1.82
2	6.36	4.79	1.07	3.02	10.50	7.33	1.81
3	5.53	4.16	1.10	3.30	10.40	7.47	1.48
4	6.18	4.85	1.61	3.00	11.10	7.90	1.77

Cell densities at the aeration changeover were 0.103 mg dry wt·ml^{-1} in the control culture and 0.099 mg dry wt·ml^{-1} in the culture which contained chloramphenicol. Intracellular cytochrome and ATP concentrations were assayed as described in the Materials and methods section.

aeration-changeover experiments (table 1) indicated that the Q_{O_2} approximately doubled prior to the resumption of growth (424 to 909 μl oxygen·hr^{-1}·mg dry wt cells^{-1}) and that over 80% of the increase occurred during the first 2 hr exposure.

Examinations of whole cell suspensions by reflectance spectrophotometry (table 2, Expt. A) indicated that the changeover from low to extra-high aeration caused no significant change in the concentration of the *b*- or *c*-type cytochromes, but induced some increase in the concentration of the cytochrome oxidase a_2. Rather unexpectedly, in view of the large increase in Q_{O_2}, the intracellular ATP concentration fell by over 40% during this period.

Phosphorylating respiratory membranes, prepared from cells harvested at hourly intervals following the changeover from low to extra-high aeration, were examined with respect to their electron transfer and energy conservation properties (table 3). P/O ratios with NADH as substrate decreased considerably over the 4 hr lag period whereas those associated with the oxidation of NADPH or malate (NAD(P)$^+$-independent) were very much less affected. These results clearly suggested that the increased aeration caused a loss of phosphorylation efficiency at the level of NADH dehydrogenase (site I). During this aeration-induced lag period the activities of the rate-limiting NADH and NADPH dehydrogenase increased by over 50% and the concentration of cytochrome a_2 increased by 83%, but no significant increases were observed in the activity either of malate dehydrogenase or of the central ubiquinone-b_1 region of the respiratory chain (as assayed from the oxygen uptake rate with the combined substrates NADH *plus* malate; see [12]).

Table 3

The effect on the properties of *A. vinelandii* respiratory membranes of changing the growth conditions from low to extra-high aeration.

Time after aeration changeover (hr)	P/O ratios			Respiratory activities (μg atom oxygen·min^{-1} nmole b_1^{-1})				Cytochrome a_2 (nmole·nmole b_1^{-1})
	NADH	NADPH	malate	NADH	malate	NADH + malate	NADPH	
0	0.76	0.43	0.51	2.01	2.05	3.56	1.91	0.21
1	0.59	0.40	0.36	2.26	2.26	3.88	2.17	0.25
2	0.55	0.43	0.43	2.62	2.08	3.82	2.37	0.28
3	0.46	0.41	0.37	2.66	2.18	4.08	3.06	0.37
4	0.52	0.36	0.44	3.08	2.00	3.92	3.12	0.39

The cell density at the aeration changeover was 0.128 mg dry wt·ml^{-1}. Phosphorylating respiratory membranes were prepared and assayed by essentially standard procedures (see Materials and methods). The rate of oxygen uptake with NADH, malate or NADH + malate was assayed under state III conditions (+ ADP and inorganic phosphate), but with NADPH was assayed under uncoupled conditions (+ 30 μM m-Cl CCP).

Table 4

The effect on the properties of *A. vinelandii* respiratory membranes of changing the growth conditions from low to extra-high aeration in the presence of chloramphenicol (200 μg·ml^{-1}).

Time after aeration changeover (hr)	P/O ratios		Respiratory activities (μg atom oxygen·min^{-1}·nmole b_1)				Cytochrome a_2 (nmole·nmole b_1^{-1})
	NADH	malate	NADH	malate	NADH + malate	NADPH	
0	0.89	0.51	1.76	1.27	3.01	2.33	0.20
1	0.82	0.54	1.88	1.47	3.05	2.30	0.20
3	0.62	0.46	1.93	1.13	3.03	2.82	0.18
4	0.62	0.59	1.71	0.93	2.87	2.77	0.22

Chloramphenicol was added to the low aeration culture to a final conc. of 200 μg·ml^{-1} and extra-high aeration conditions were initiated, 1 hr later, at a cell density of 0.103 mg dry weight·ml^{-1}. Phosphorylating respiratory membranes were prepared and assayed as for table 3.

In order to determine whether the observed changes reflected activation, inhibition or *de novo* synthesis of certain respiratory chain components, these experiments were repeated following the addition of chloramphenicol, a potent inhibitor of protein synthesis [13], to the low aeration cultures 1 hr prior to the changeover to extra-high aeration conditions. Chloramphenicol significantly decreased the extent of the aeration-induced increase in Q_{O_2} (table 1) and largely prevented the increase in the intracellular concentration of cytochrome a_2 (table 2, Expt. B). At the respiratory membrane level chloramphenicol completely abolished the increase in NADH dehydrogenase activity, substantially abolished the increase in NADPH dehydrogenase activity and cytochrome a_2 concentration, but appeared to have no effect on the loss of site I phosphorylation (table 4).

The *in vitro* exposure of well-coupled respiratory membranes from *A. vinelandii* to highly aerobic conditions (by rapid stirring under air for up to 2 hr at 25°) had no effect on their energy conservation or electron transfer properties over and above some slight, unspecific loss of dehydrogenase activity which was also exhibited by control preparations (stirred under argon). Similarly, chloramphenicol (200 μg·ml^{-1}) had no significant effect on oxygen uptake or ATP synthesis associated with the oxidation of NADH, NADPH or malate by isolated respiratory membranes when added either to the isolated membranes 1 min prior to assay or to low aeration parent cultures 4 hr prior to harvesting.

4. Discussion

The results described in this paper clearly indicate that the aeration-induced enhancement of respiratory activity, which is characteristic of nitrogen-fixing cultures of *Azotobacter* spp. and which serves to protect nitrogenase from the deleterious effects of molecular oxygen [3], is accompanied by significant changes in the membrane-bound respiratory system, *viz.*

i) increased NADH and NADPH dehydrogenase activity;

ii) increased concentration of cytochrome oxidase a_2; and

iii) loss of energy conservation at site I.

The failure of isolated *A. vinelandii* respiratory membranes to mimic any of the above effects following exposure to highly aerobic conditions clearly rules out direct activation or inhibition by molecular oxygen.

Since chloramphenicol largely abolishes the increase in both reduced pyridine nucleotide dehydrogenase activity and cytochrome a_2 concentration, as well as severely damping the Q_{O_2} increase (in spite of an earlier preliminary report to the contrary [7]), it appears likely that the *de novo* synthesis of selected respiratory chain components is necessary for at least part of the total increase in respiratory activity. The requirement for more of the major, but rate-limiting, dehydrogenases is not surprising but the concomitant synthesis of non-limiting terminal oxidase is initially rather puzzling, (see also [5]). However, recent work in this laboratory (D.J. Meyer and C.W. Jones, unpublished data) indicates that the co-ordinate synthesis of cytochrome oxidases a_1 and a_2 causes a decrease in the whole cell oxidative phosphorylation efficiency of *E. coli* W; preliminary work on *A. vinelandii* suggests that these two oxidases may also terminate respiratory chains of low energy conservation efficiency in this organism.

The aeration-induced loss of site I is not unexpected considering its low efficiency in early logarithmic phase cells [7], but the mechanism via which this loss occurs remains unclear. Since highly aerobic conditions lead to the "switching-off" of nitrogenase [4, 5] and hence to an increased availability of NADPH for oxidation via the non-phosphorylating NADPH dehydrogenase [8], excess oxygen clearly promotes the utilisation of respiratory pathways of relatively low energy conservation efficiency in *A. vinelandii*.

The respiratory activity of an aerobe, in the absence of growth, is that due solely to the maintenance respiration, which has been defined by Harrison and Loveless [14] as M/N where M is the maintenance coefficient (moles ATP consumed·hr^{-1}·g $cells^{-1}$) and N is the phosphorylating efficiency (moles ATP produced·mole oxygen $consumed^{-1}$; i.e., P/O ratio × 2). Thus, for the observed doubling of the *A. vinelandii* Q_{O_2} to occur, either M must increase and/or N must decrease. There is no evidence to support the former possibility but there is certainly evidence to suggest that the latter occur, either M must increase and/or N must decrease. and via the increased use of cytochrome oxidase a_2 and the non-phosphorylating NADPH dehydrogenase.

It is likely that the respiratory system of *A. vinelandii* has attained its characteristic complex and highly branched nature [15, 16] to enable the organism to vary readily its efficiency of energy conservation, and hence its respiratory activity, in order to combat sudden, potentially dangerous changes in its growth environment.

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