RESPIRATION-LINKED PROTON TRANSLOCATION IN *AZOTOBACTER VINELANDII*

A. J. DOWNS and C. W. JONES

Department of Biochemistry, School of Biological Sciences, University of Leicester, University Road, Leicester LE1 7RH, England

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1. Introduction

Azotobacter vinelandii is a Gram negative, obligate aerobe which reductively assimilates atmospheric nitrogen via an oxygen-sensitive nitrogenase complex [1,2]. The organism if thus faced with the problem of maintaining its intracellular oxygen concentration at a level which is too low to inhibit nitrogenase activity, yet high enough to allow oxidative phosphorylation. Clearly, this regulation must be manifested via its respiratory system which therefore has a dual function, viz the synthesis of ATP and the removal of excess oxygen.

The *Azotobacter* respiratory chain is characterised by an extremely rich complement of redox carriers, a highly active NADPH dehydrogenase (*plus* the usual dehydrogenases) and a branched terminal cytochrome system ([3–7]; fig.1.). Experiments with isolated respiratory membranes have indicated the presence of three energy coupling sites, viz at the level of NADH dehydrogenase (site I), the central Q → *b* region (site II) and the minor, cyanide-sensitive branch of the terminal cytochrome system (site III); the major cytochrome branch appears to be uncoupled [8–10]. Energy conservation at site I is absent from membranes prepared from cells grown at very high oxygen concentrations [8,11]. The *Azotobacter* respiratory system would thus be capable of considerable flexibility with respect to its energy conservation efficiency according to the exact electron transfer pathways employed. Since the respiratory activity of a growing culture is inversely related to the energy conservation efficiency [12], the organism is apparently able to regulate its respiratory activity (and hence its intracellular oxygen content) largely at the dictate of the ambient oxygen concentration. This process has been called respiratory protection [13].

This hypothesis is open to the criticism that it is based predominantly upon the results of P/O ratio measurements using poorly coupled membrane preparations from *Azotobacter*. Furthermore, it is possible that some of the data upon which it is based reflect changes in energy coupling which are caused by the isolation procedures rather than by genuine physiolo-

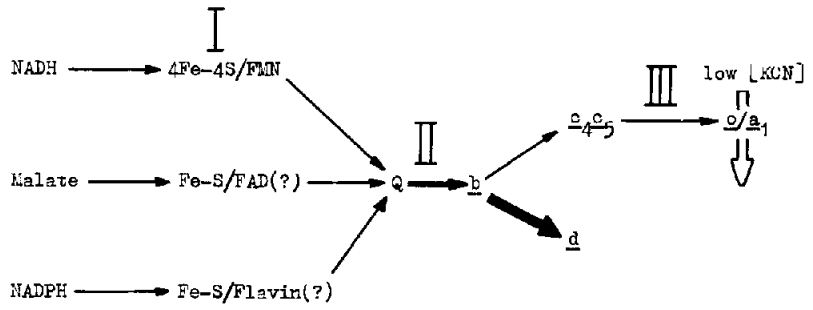

Fig.1. The respiratory system of *A. vinelandii*. Solid arrows represent pathways of electron transfer, open arrows represent the site of action of respiratory chain inhibitors.

gical events. It is therefore imperative that these results should be verified, or otherwise, using whole cells (where these objections should no longer apply).

Of the various methods available for measuring oxidative phosphorylation in whole cells the simplest and most reliable is the indirect method based upon Mitchell's chemiosmotic theory of oxidative phosphorylation [14], viz the measurement of respiration-linked proton translocation. In this paper we report measurements of $\rightarrow H^+/O$ ratios in whole cells of *A. vinelandii*, the results of which confirm and extend the above hypothesis concerning the dual role of the respiratory system in this organism.

2. Materials and methods

2.1. *Culture conditions*

Azotobacter vinelandii (NCIB 8660) was grown in batch culture at 30°C under high aeration, nitrogen-fixing conditions as described previously [3,8]. Unless otherwise stated, cells were harvested either in the early stages of logarithmic growth ($A_{680} \leqslant 0.4, [O_2] \ll 150\ \mu M$; denoted excess oxygen cells) or during the post-logarithmic growth phase ($A_{680} \geqslant 2.5, [O_2] < 2\ \mu M$; denoted oxygen-limited cells).

2.2. *$\rightarrow H^+/O$ ratios*

Whole cell suspensions were prepared and assayed for respiration-linked proton translocation by essentially procedures [15,17], but with the following modifications to the assay mixture; pH 6.9 ± 0.1, cell density 6.0 mg dry wt·ml^{-1}, 100 mN KSCN and 0.1 mg·ml^{-1} carbonic anhydrase. The stoichiometry of proton translocation is expressed as the $\rightarrow H^+/O$ ratio (gequiv H^+·g atom O^{-1} consumed, [14]). For the measurements of $\rightarrow H^+/O$ ratios for the oxidation of exogenous substrates, cells were starved of endogenous substrates as described previously [17] and were then incubated with 10 mM β-hydroxybutyrate, isocitrate or L-malate, and 12 mM ascorbate *plus* 7.6 mM TMPD* or DCPIP.

2.3. *Miscellaneous*

Dissolved oxygen concentrations and respiratory activities of growing cultures, and of washed cells ± added substrates, were assayed by standard procedures [9,11,17]. Potassium cyanide was freshly prepared for each batch of experiments.

* *Abbreviations*: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride; DCPIP, 2,6-dichlorophenol indophenol.

3. Results and discussion

According to the chemiosmotic hypothesis of oxidative phosphorylation [14], the respiratory chain is organised into a series of redox loops, each of which is able to translocate 2 protons outwards per pair of reducing equivalents transferred. The $\rightarrow H^+/O$ ratio is therefore equal to *twice* the P/O ratio, assuming an $\rightarrow H^+/P$ ratio of 2 (as is the case with mitochondria [18]). Sites I, II and III are therefore viewed as loops 1, 2 and 3 respectively, each of which contributes to the total transmembrane protonmotive force which drives ATP synthesis [14].

Measurements of respiration-linked proton translocation in whole cells of *A. vinelandii* confirmed that respiration was accompanied by the rapid extrusion of protons. Analysis of the decay kinetics of the proton gradient indicated that it was first order with respect

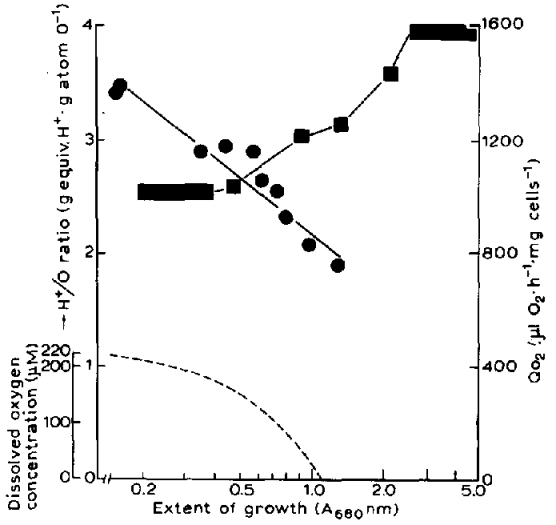

Fig.2. The variation of dissolved oxygen concentration, endogenous $\rightarrow H^+/O$ ratio and respiratory activity (Qo_2) during the growth of *A. vinelandii* in batch culture. These parameters were assayed as described in the Materials and methods section. Dissolved oxygen concentration (- - -), Qo_2 (●) and $\rightarrow H^+/O$ ratio. (■) The values for excess oxygen and oxygen-limited cultures are taken from Table 1).

Table 1
$\rightarrow H^+/O$ ratios for the oxidation of endogenous substrates by whole cells of *A. vinelandii*

Inhibitor	$\rightarrow H^+/O$ ratio		$\Delta \rightarrow H^+/O$ ratio
	Excess oxygen cells	Oxygen-limited cells	(oxygen-limited *minus*-excess oxygen cells)
	(g equiv $H^+ \cdot$g atom O^{-1}		
–	2.53 ± 0.15 (6)	3.94 ± 0.16 (7)	1.41
KCN (50 μM)	2.10 ± 0.09 (5)	3.24 ± 0.13 (5)	1.14
$\Delta \rightarrow H^+/O$ (± KCN)	0.43	0.70	

$\rightarrow H^+/O$ ratios were assayed as described in the Materials and Methods section. Results are expressed as the average ± standard error of the mean, with the number of determinations in brackets.

to protons and exhibited an average $t_{\frac{1}{2}}$ of approx. 50 seconds.

During the growth of *A. vinelandii* in batch culture, $\rightarrow H^+/O$ ratios for the oxidation of endogenous substrates increased from approx. 2.5 in early logarithmic phase, excess oxygen cells to reach a maximum value of just under 4 after the cells became oxygen-limited (fig.2; see also table 1). This behaviour was very similar to that which was observed earlier for the P/O ratios of respiratory membranes oxidising NADH [9]. The increase in energy conservation efficiency was accompanied by a decrease in the Qo_2 of the growing culture, i.e. the latter appears to be inversely related to the former as predicted by Harrison and Loveless [12] for other bacteria.

Further analysis of endogenous $\rightarrow H^+/O$ ratios in cells harvested from the excess oxygen and oxygen-limited stages of growth involved the use of 50 μM cyanide which strongly inhibits only the cytochrome $c_4c_5 \rightarrow a_1o$ terminal pathway [4,7]. Cyanide significantly decreased the $\rightarrow H^+/O$ ratios of both types of cells (table 1) thus indicating that this branch, rather than the cytochrome $b \rightarrow d$ pathway, is associated with energy conservation at the level of loop 3. Since this decrease was considerably less than half of the maximum value for one loop (2 g equiv $H^+ \cdot$g atom 0^{-1}), it is likely that the respiratory activity of the energy-conserving cytochrome $c_4c_5 \rightarrow a_1o$ branch is less than that of the uncoupled cytochrome $b \rightarrow d$ pathway under uninhibited conditions. The $\rightarrow H^+/O$ ratio of 2.10 which was observed with excess oxygen cells *plus* cyanide suggests that only one proton translocating loop is active under these conditions (probably loop 2). In contrast, oxygen-limited cells exhibited an $\rightarrow H^+/O$ ratio of 3.23 in the presence of cyanide, thus confirming the appearance of an extra protontranslocating loop during growth at low oxygen concentrations; this is probably loop 1.

The foregoing experiments were carried out using cells which oxidised endogenous substrates of ill-defined composition, thus making a detailed interpretation of the results rather hazardous. In an attempt to eliminate this problem, cells were starved as extensively as possible and then loaded with specific, exogenous substrates. The effect of starvation on proton-translocation was clearly to decrease the $\rightarrow H^+/O$ ratios of oxygen-limited cells to the values which were observed with excess oxygen cells (starved or unstarved), i.e. approximately 2.5 equiv $H^+ \cdot$g atom O^{-1} (table 2). This apparently basal, physiological value was not substantially altered following the loading of either type of cells with malate or isocitrate, both of which are oxidised independently of NAD^+, i.e. via flavin- and $NADP^+$-linked dehydrogenases respectively [3,6]. In contrast, loading with the NAD^+-linked substrate β-hydroxybutyrate significantly increased the $\rightarrow H^+/O$ ratios of oxygen-limited cells, although it had absolutely no stimulatory effect on excess oxygen cells. The former response was considerably less than was expected (viz, an $\rightarrow H^+/O$ ratio $\geqslant 4.0$), probably because it was impossible to totally abolish endogenous respiration, even by prolonged starvation, and because β-hydroxybutyrate stimulated this basal respiration rate only very slightly. Nevertheless, if the observed $\rightarrow H^+/O$ ratio of 2.83 is corrected for endogenous proton translocation (see [19]), the oxidation of β-hydroxybutyrate alone yields an $\rightarrow H^+/O$ ratio of 3.51. This value thus supports the presence of loop 1 in oxygen-limited cultures of

Table 2

$\rightarrow H^+/O$ ratios and respiratory activities of starved whole cells of *A. vinelandii* oxidising exogenous substrates

Substrate	Immediate electron acceptor	Excess oxygen cells		Oxygen-limited cells	
		$\rightarrow H^+/O$ ratios (g equiv $H^+ \cdot$ g atom O^{-1})	Qo_2 (μl O_2 $mg^{-1} \cdot h^{-1}$)	$\rightarrow H^+/O$ ratios (g equiv $H^+ \cdot$ g atom O^{-1})	Qo_2 (μl $O_2 \cdot mg^{-1}$ h^{-1}
Endogenous	–	2.42 ± 0.09 (4)	28.6 ± 7.0 (4)	2.46 ± 0.16 (11)	9.4 ± 1.7 (9)
+ β-hydroxybutyrate	NAD^+	2.17 ± 0.13 (4)	32.1 ± 7.8 (4)	2.83 ± 0.13 (6)	14.6 ± 2.3 (9)
+ isocitrate	$NADP^+$	2.33 ± 0.07 (3)	41.1 ± 6.6 (4)	2.21 ± 0.13 (3)	12.6 ± 2.1 (8)
+ malate	Flavin	2.11 ± 0.05 (4)	83.9 ± 15.6 (4)	2.38 ± 0.20 (7)	38.9 ± 7.5 (8)
+ ascorbate-DCPIP	c_4c_5			1.74 ± 0.24 (3)	22.1 ± 4.4 (6)
+ ascorbate-TMPD	c_4c_5			1.60 ± 0.25 (4)	62.2 ± 8.2 (7)

$\rightarrow H^+/O$ ratios and respiratory activities (Qo_2) of starved cells ± exogenous substrates were assayed as described in the Materials and methods section. Values are expressed as the average ± standard error of the mean, with the number of determinations in brackets.

A. vinelandii, although this loop is clearly absent in excess oxygen cells.

The $\rightarrow H^+/O$ ratios of 1.60 and 1.74 for the oxidation of reduced TMPD and DCPIP respectively confirmed the presence of loop 3 on the cytochrome c_4c_5-linked terminal branch. Oxygen uptake (and hence proton translocation) with these two artificial substrates was totally abolished by 50 μM cyanide. In contrast, the oxidation of exogenous *physiological* substrates was largely unaffected by the addition of cyanide although their $\rightarrow H^+/O$ ratios (not shown in table 2) decreased to approximately 2 g equiv $H^+ \cdot$g atom O^{-1}; this value is compatible with the utilisation of loop 2 only. The one exception was the oxidation of β-hydroxybutyrate by oxygen-limited cells where the $\rightarrow H^+/O$ ratio in the presence of cyanide was greater than 2.5, thus adding further support to the presence of loop 1.

These results support the concept that the respiratory system of *A. vinelandii* contains one basic, energy conserving loop (loop 2) which can be supplemented by the cytochromes $c_4c_5 \rightarrow a_1o$ pathway (loop 3). The location of the latter is fully supported by our results with other bacteria [17], from which it is apparent that loop 3 is present only when a *c*-type cytochrome is available to act as its electron carrier. Clearly, the overall efficiency of energy conservation in the terminal cytochrome system of *A. vinelandii* depends upon the relative activities of the $c_4c_5 \rightarrow a_1o$ branch and the uncoupled $b \rightarrow d$ pathway. The $\rightarrow H^+/O$ ratios observed with *A. vinelandii* do not support the view [2,8] that the $c_4c_5 \rightarrow a_1o$ branch is further split into a slow, energy-conserving pathway *plus* an active, uncoupled pathway.

Only under oxygen-limited conditions are loops 2 and 3 supplemented by loop 1. When the latter is present, the overall efficiency of energy conservation is of course dependent upon the activity of NADH dehydrogenase relative to that of the uncoupled malate and NADPH dehydrogenases.

Assuming an $\rightarrow H^+/P$ ratio or 2, the respiratory system of *A. vinelandii* thus exhibits a maximum endogenous P/O ratio of slightly under 2. This value is in good agreement with the results of direct measurements by Knowles and Smith [20] (but see also [21]) and suggests that NADH is the major endogenous substrate. The observed variation in P/O ratio from a minimum of 1.0 up to at least 2.0, according to the exact conditions of growth and to the availability of oxidisable substrates, confirms and extends our earlier work with isolated membranes and fully supports the concept of respiratory protection in this organism.

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