SOME PARAMETERS AFFECTING RESPIRATORY CONTROL IN *AZOTOBACTER VINELANDII* MEMBRANES

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1. Introduction

Respiratory control (the stimulation of respiration by ADP + Pi, followed by a return to the original rate on the exhaustion of either cofactor) has been previously demonstrated in animal and plant mitochondria [1, 2] and, more recently, in isolated respiratory membranes from *Azotobacter vinelandii* [3–5] and *Micrococcus denitrificans* [6].

Respiratory membranes from *A. vinelandii* rapidly oxidise NADH and malate (not via $NAD(P)^+$) with quite high phosphorylation efficiencies (P/O, NADH = 0.90–1.10; [7]). The major sites of energy coupling are at NADH dehydrogenase (I) and in the region of ubiquinone between the primary dehydrogenases and the terminal cytochrome system (II). Site III is located on a highly KCN-sensitive, minor branch of the terminal cytochrome system [8] and contributes little to the total energy coupling. Hence NADH oxidation is coupled at sites I and II and malate at site II only ([9]; see also fig. 1).

This paper describes several parameters which affect respiratory control at sites I and II in partly coupled membranes from *A. vinelandii*.

2. Materials and methods

Azotobacter vinelandii (NCIB 8660) was cultured at high aeration on N_2-mannitol medium [10], harvested, suspended in 10 mM PIPES buffer pH 6.4 containing 8 mM Mg acetate and disintegrated in a French pressure cell (American Instrument Co., Silver Spring, Maryland, USA) at 4,000 lb/in^2. The cell free extract (28,000 *g*, 15 min) was centrifuged at 59,000 *g*, 15 min to sediment the phosphorylating respiratory membranes.

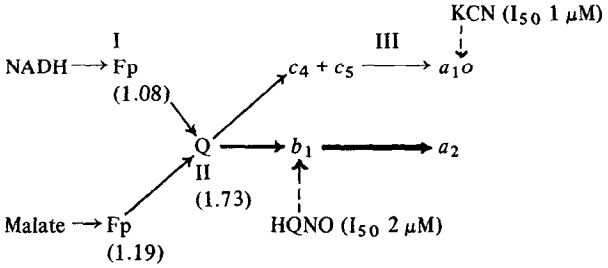

Fig. 1. The respiratory system of *Azobacter vinelandii*. Inhibitor action is indicated by the broken arrows. The numbers in brackets refer to state 4 electron transfer rates (see table 1); I, II, III represent the three energy coupling sites.

Oxygen uptake and $AT^{32}P$ synthesis were assayed by essentially standard procedures ([9–11], table 1); P/2e at site I was estimated from the P/O NADH *minus* P/O malate [9]. Protein was determined by the modified biuret method [12].

Respiratory states 3 and 4 were as defined by Chance and Williams [13]. The terminal respiratory pathway was defined as the segment of the respiratory chain between oxygen and the entry of reducing equivalents from the separate dehydrogenases.

3. Results

The oxidation of NADH by partly coupled mem-

Abbreviations:
RCI : respiratory control index (state 3/state 4 rate of oxygen uptake)
m-Cl CCP: carbonylcyanide *m*-chlorophenyl hydrazone
HQNO : 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide
PIPES ; piperazine-*N*,*N*′-bis 2-ethane sulphonic acid

branes was maximally stimulated by the addition of ADP (K_m = 7.2 μM) *plus* Mg^{2+} (K_m = 90 μM) *plus* Pi, or of the uncoupling agent m-Cl CCP (> 30 μM). When limiting amounts of ADP were used, in the presence of Pi, respiration was slowed to the controlled rate (state 4) on the exhaustion of the ADP but could again be stimulated to the maximum rate (state 3) by the further addition of ADP or m-Cl CCP. Mg^{2+}, Pi, AMP, ADP or ATP alone were without effect.

High respiratory control indices (RCI = 1.40–1.60) were observed with NADH provided that site I energy coupling was high (P/2e = 0.36; table 1), but not with malate (RCI < 1.05) in spite of the relatively high energy coupling at site II (P/2e = 0.43). The RCI with NADH *plus* malate was little altered from that with NADH alone (10 separate membrane preparations yielded an average increase of 0.03) although malate must have supplied at least one-third of the reducing equivalents and might therefore have been expected to substantially decrease the RCI.

The effect of using the combined substrates was to shift the rate-limiting step of electron transfer from the primary dehydrogenases to some point in the terminal system and thus to increase the dehydrogenase/terminal pathway activity ratio (table 1). The use of HQNO at increasing concentrations (up to 2 μM) to progressively inhibit the major cytochrome b_1-linked terminal respiration pathway [8, 9] similarly increased this ratio and resulted in a linear increase in the RCI with malate (fig. 2a). This RCI increase was not affected by 10 μM KCN, thus eliminating involvement of site III.

With NADH as substrate (fig. 2b) an HQNO-induced increase in the RCI was observed only with membranes which exhibited poor energy coupling at site I (prepared from cells harvested in very early logarithmic growth) and hence a low basal RCI. In membranes with high energy coupling at site I HQNO caused a slight decline in the high basal RCI. The latter probably reflected the weak uncoupling activity of inhibitor [9, 14, 15] and suggested that the increases in RCI described above were probably slight underestimates.

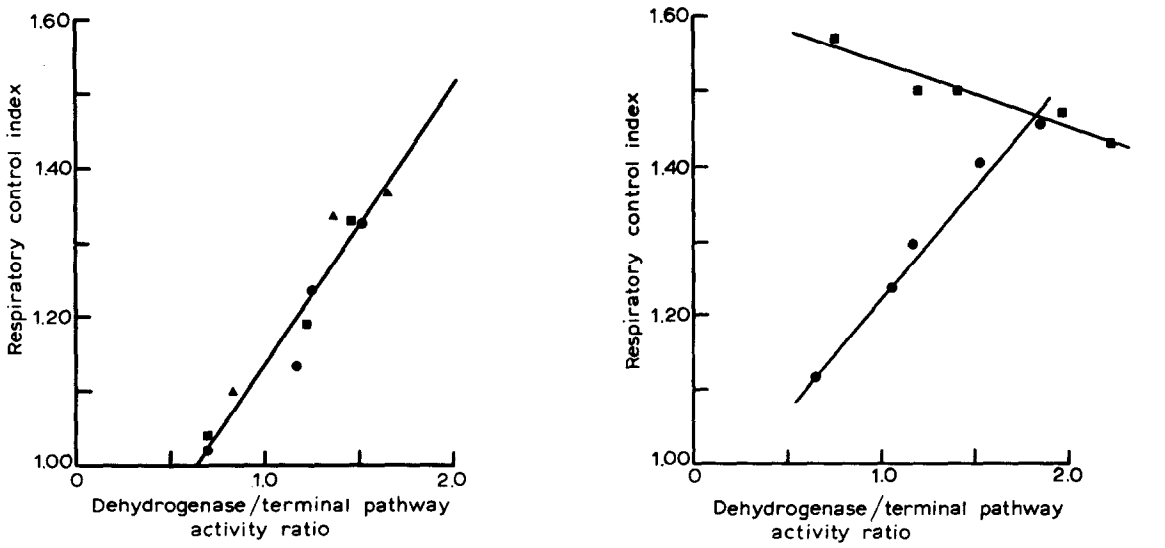

Fig. 2. The effect on the respiratory control index of increasing the dehydrogenase/terminal pathway activity ratio with HQNO. The reaction conditions were as described for table 1; 0.15–0.25 mg respiratory membrane protein/assay. A maximum HQNO concentration of 2.0 μM was employed; above this concentration the RCI began to decrease.
(a) Malate; three separate membrane preparations (–●–■–▲–); P/2e site II 0.38–0.43.
(b) NADH; P/2e site I 0.08, site II 0.33 (–●–); P/2e site I 0.30, site II 0.38 (–■–).

Table 1
Respiratory control indices of *Azotobacter vinelandii* membranes.

Substrate	Oxidase activities (μatoms O/min/mg protein)		RCI (State 3/4)	Dehydrogenase/terminal pathway activity ratio (state 4)
	State 4	State 3		
NADH	1.08	1.53	1.41	0.63
Malate	1.19	1.21	1.02	0.69
NADH + malate	1.73	2.32	1.38	1.32

The reaction mix contained 42 mM PIPES buffer pH 6.8, 8 mM magnesium acetate, 2.1 mM Na/K phosphate and 0.23 mg respiratory membrane protein (state 4) *plus* 1.0 mM ADP (state 3). Final volume 2.0 ml; temperature 30°. The reaction was started by the addition of 1.0 mM NADH and/or 7.5 mM malate. P/2e values: site I 0.36, site II 0.43. The terminal pathway activity was taken to be the observed saturating rate with NADH *plus* malate.

4. Conclusions

The data described in this paper indicate that respiratory control in partly coupled membranes from *A. vinelandii* is determined by at least three parameters:

1) For significant respiratory control to be detectable an energy coupling site must be located coincident with the rate-limiting step of electron transfer. Thus high respiratory control indices are observed with NADH (site I), also with NADH *plus* malate and with malate *plus* HQNO (site II), but not with malate alone.

2) The electron pressure at the controlling site partly determines the degree of respiratory control. Hence with malate, and also under certain conditions with NADH, the RCI increases linearly as a function of the increase in electron pressure at site II (measured as the dehydrogenase/terminal pathway activity ratio).

3) The efficiency of energy coupling at the controlling site also partly determines the degree of respiratory control. The low RCI observed with NADH when energy coupling at site I is low can therefore be stimulated by shifting control to an efficiently coupled site II.

It is likely that the previous failure of many partly coupled bacterial membrane preparations [16] to exhibit respiratory control stems from an inability to satisfy one or more of the above parameters.

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