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# RESPIRATORY CONTROL IN *AZOTOBACTER VINELANDII* MEMBRANES

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SUMMARY

1. Significant respiratory control was observed with partly coupled respiratory membranes of *Azotobacter vinelandii* only when an energy conservation site was coincident with a rate-limiting step in electron transfer. This criterion was satisfied with NADH alone (Site I), NADH *plus* malate (Site II) but not with malate alone. No respiratory control was observed at Site III.

2. P/*2e* ratios (Sites I and II) and relative activities of different segments of the respiratory chain were found to vary as a function of high aeration growth.

Using respiratory membranes prepared from cells harvested at carefully determined stages of high aeration growth, respiratory control indices (+ADP/—ADP) were demonstrated to be proportional both to the efficiency of energy coupling at the controlling site (assayed as the P/*2e* ratio) and to the extent of rate-limitation in electron transfer at that site (determined from the relative electron transfer activities of different segments of the respiratory chain). Maximum respiratory control indices of 1.65 and 1.55 were observed at Site I and II, respectively.

3. These results indicate that the ability of isolated *Azotobacter vinelandii* respiratory membranes to exhibit respiratory control is influenced by the whole cell growth environment.

## INTRODUCTION

Respiratory control (defined as the stimulation of respiration by ADP *plus* inorganic phosphate followed by a return to the original controlled rate on the exhaustion of ADP)[1] has been widely demonstrated in animal and plant mitochondria (for examples see refs. 2, 3). An oligomycin-induced, uncoupler-dependent respiratory control has been demonstrated for submitochondrial particles[4].

Uncoupler-mediated respiratory control has also been demonstrated with whole cells of certain bacteria[5–7] and increased rates of oxygen uptake in the presence of inorganic phosphate or phosphate acceptor have been reported for some subcellular preparations[8–10]. Not until very recently, however, has a classical, mitochondrial type of respiratory control been reported for isolated bacterial membranes (*viz.* those of

Abbreviations: PES, piperazine-*N*,*N*′-bis-2-ethanesulphonic acid; TMPD, tetramethyl-*p*-phenylenediamine; DCIP, 2,6-dichlorophenolindophenol.

*Azotobacter vinelandii*[11,12] and *Micrococcus denitrificans*[13]) in spite of the widely reported ability of many bacterial preparations to catalyse oxidative phosphorylation (see ref. 14).

The membrane-bound respiratory system of *Azotobacter vinelandii*, a Gram negative, nitrogen-fixing aerobe, is characterised by its extremely high activity and by the presence of a complex, branched cytochrome system[15–19] (Fig. 1). Carefully prepared respiratory membranes rapidly oxidise NADH or malate (not *via* pyridine nucleotide) with reasonably high energy coupling efficiency (P/O, NADH $\leqq$ 1.10)[20]. Major phosphorylation sites (P/2*e* $\leqq$ 0.5) are associated with NADH dehydrogenase (Site I)[11,20] and with the region of the respiratory chain between the cytochrome system and the entry of reducing equivalents from the dehydrogenases (*i.e.* Q; Site II). Site III (P/2*e* $\leqq$ 0.23) is located on the highly KCN-sensitive minor branch of the respiratory system and contributes little to the total energy coupling[20]. Hence the oxidation of NADH is coupled at Sites I and II and malate at Site II only.

The complex nature of the respiratory NADH dehydrogenase of *A. vinelandii* is reflected by the action of juglone (5-hydroxy naphthoquinone) which appears to accept electrons very rapidly ($\geqq$ 2 $\mu$moles NADH/min per mg protein) from an electron carrier (X; see Fig. 1)) located on the substrate side of the energy coupling site (S. K. ERICKSON, B. A. C. ACKRELL AND C. W. JONES, unpublished data). This conclusion is based on the absence both of concomitant ATP synthesis and of respiratory control. On the other hand electron transfer over the complete NADH dehydrogenase segment is considerably slower (typically, 1.08 $\mu$moles NADH/min per mg protein) and exhibits both ATP synthesis (P/2*e* $\leqq$ 0.5)[20] and respiratory control[12]. The most likely location of Site I is therefore between the postulated, but as yet unidentified, components X and Y of NADH dehydrogenase.

This paper reports the results of investigations into the nature of respiratory control in partly coupled membranes of *A. vinelandii* and describes how this phenomenon is influenced by several environment-controlled properties of these membranes.

## MATERIALS AND METHODS

### *Chemicals*

NADH, ADP, piperazine-*N*,*N*′-bis- 2-ethanesulphonic acid (PES) and juglone were obtained from Sigma, St. Louis, Mo., U.S.A. All other chemicals were obtained from British Drug Houses, Poole, Dorset, England and were of the finest grade available. Double glass-distilled water was used throughout this work.

### *General preparative procedures*

*A. vinelandii* (N.C.I.B. 8660) was cultured at 30° on $N_2$–mannitol medium at high aeration (14.2 mmoles $O_2$ dissolved/h per l) as described previously[16,20]. Unless otherwise stated cells were harvested at the turnover point from logarithmic into oxygen-limited, non-logarithmic growth ($A_{680\,nm} \geqq 1.05$) suspended in 10 mM PES buffer (pH 6.4) containing 8 mM magnesium acetate and broken in a French pressure cell (American Instrument, Silver Spring, Md., U.S.A.) at 4000 lb/inch$^2$. The broken cell suspension was centrifuged at 28000 $\times$ *g* for 15 min to yield the cell free extract which in turn was centrifuged at 59000 $\times$ *g* for 15 min to sediment the phosphorylat-

ing respiratory membranes. The latter were resuspended in PES–magnesium acetate (pH 6.4) and used immediately.

*General assay procedures*

Oxygen uptake was followed at 30° in a Clark electrode using a reaction medium containing 42 mM PES buffer (pH 6.8), 8 mM magnesium acetate, 2.1 mM $Na_2HPO_4$–$KH_2PO_4$ buffer (pH 6.8) and approx. 0.25 mg respiratory membrane protein (— ADP) *plus* 1 mM ADP (+ ADP); final volume 2.0 ml. The reaction was initiated by the addition of 1 mM NADH, 7.5 mM malate or a mixture of the two substrates. The respiratory control index was calculated from the ratio of the +ADP/—ADP oxygen uptake rates.

Oxygen uptake was also routinely used as an assay for the NADH and malate dehydrogenase segments of the respiratory chain since the latter were rate limiting for NADH and malate oxidases respectively, (see Table I later).

P/O ratios were determined using the above reaction mixture (final volume 4.0 ml) supplemented with 4.2 mM glucose, 45 units of yeast hexokinase (Sigma type III), 1.7 mg bovine serum albumin and $^{32}P_i$ to give a specific activity of approx. $1.0 \cdot 10^4$ counts/min per μmole phosphate[20]. 3-ml samples were removed into 1 ml ice-cold trichloroacetic acid (10%, v/v) 1 min after the addition of substrate and assayed for [$^{32}$P]ATP by the method of AVRON[22]. A no-substrate blank was used as a control. P/2*e* ratio at Site I was calculated from the P/O NADH *minus* P/O malate, and the P/2*e* at Site II was taken to be the P/O with malate alone[20].

NADH–juglone reductase activity was assayed from the rate of oxygen uptake *via* reduced juglone autoxidation on the addition of 0.28 mM juglone to membranes where the NADH oxidase activity was completely inhibited by 1 mM KCN.

Protein was determined by the modified Biuret method of GORNALL *et al.*[23].

*Nomenclature*

NADH and malate dehydrogenases were defined as those segments of the respiratory chain which transfer reducing equivalents from the respective substrates into the quinone–cytochrome system (*i.e.* they may contain more than one electron transfer component).

The terminal respiratory pathway was defined as the segment of the respiratory chain between oxygen and the entry of reducing equivalents from the dehydrogenases; the rate-limiting step of this pathway was assayed as the observed rate with the combined substrates NADH *plus* malate, *i.e.* with both substrates present simultaneously.

The dehydrogenase/terminal pathway activity ratio was calculated with NADH, for example, using the respiratory activities quoted in Table I to be $1.08/1.73 = 0.63$, and for the combined substrates NADH *plus* malate to be $(1.08 + 1.19)/1.73 = 1.32$. These values were used as a measure of the location and extent of rate-limiting electron transfer at various points in the respiratory chain (*e.g.* values $\ll 1$ indicated severe rate limitation at the dehydrogenase level, values $\gg 1$ indicated severe rate limitation in the terminal respiratory pathway). The NADH–juglone/NADH dehydrogenase activity ratios were calculated and interpreted in a similar manner.

## RESULTS

Relatively high respiratory control indices (1.40–1.60) were routinely observed with NADH but not with malate or with the Site III electron donors[20] ascorbate–DCIP or ascorbate–TMPD (respiratory control index $\leqq$ 1.05). The respiratory control index observed with NADH *plus* malate was little altered from that with NADH alone (the average difference observed with 10 separate membrane preparations was +0.03) although, from a study of the observed oxidase activities (Table I), malate

TABLE I

RESPIRATORY CONTROL INDICES OF *A. vinelandii* MEMBRANES OXIDISING VARIOUS SUBSTRATES

Respiratory control indices, respiratory activities, P/2*e* ratios and dehydrogenase/terminal pathway activity ratios were determined as described in MATERIALS AND METHODS. P/2*e*: Site I, 0.36; Site II, 0.43.

| *Substrate* | *Oxidase activity* (*μgatoms O/min per mg protein*) | | *Respiratory control index* | *Dehydrogenase/terminal pathway activity ratio* |
|---|---|---|---|---|
| | *−ADP* | *+ADP* | (*+ADP/−ADP*) | (*−ADP*) |
| NADH | 1.08 | 1.53 | 1.41 | 0.63 |
| Malate | 1.19 | 1.21 | 1.02 | 0.69 |
| NADH *plus* malate | 1.73 | 2.32 | 1.38 | 1.32 |

must have supplied at least one-third of the reducing equivalents and might therefore have been expected to decrease the respiratory control index by a similar amount. The effect of using NADH *plus* malate *vis à vis* the individual substrates was to shift the rate-limiting step of electron transfer from the level of NADH or malate dehydrogenase to the region of the terminal respiratory system between cytochrome $b_1$ and the point of entry of reducing equivalents from the dehydrogenases, *viz.* the region of Site II phosphorylation (see Fig. 1 and Table I). The low aerobic steady state reduction of cytochrome $b_1$ ($\leqq$ 25 %) observed in the presence of NADH, malate or NADH *plus* malate further suggested that this segment was followed by a much faster $b_1 \rightarrow$ oxygen span.

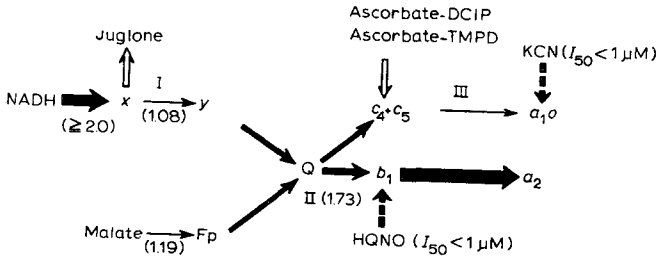


Fig. 1. The respiratory system of *A. vinelandii*. I, II, III represent the three energy coupling sites. The numbers in parentheses refer to electron transfer rates (*μ*moles substrate oxidised/min per mg protein; see Table I) in the absence of ADP. Inhibitor action is indicated by the broken arrows, physiological electron transfer by the solid arrows and artificial electron transfer by the open arrows.

The phosphorylation efficiences of isolated *A. vinelandii* respiratory membranes (assayed as P/O ratios) varied considerably, but reproducibly, with the stage of high aeration growth at which the cells were harvested (Fig. 2). P/2*e* ratios at Site I were very low during early logarithmic, excess oxygen growth but later sharply increased to reach maximum values at the turnover into non-logarithmic oxygen-limited growth ($A_{680\ nm} = 1.05$). P/2*e* ratios at Site II increased much more rapidly over the early stages to reach maximum values well before the end of logarithmic growth ($A_{680\ nm} = 0.7$).

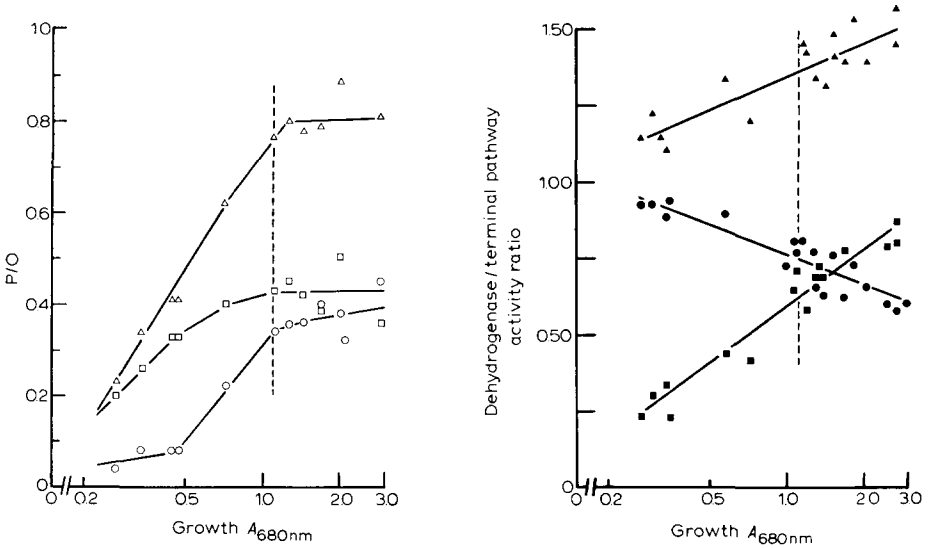


Fig. 2. Phosphorylation efficiences of isolated respiratory membranes as a function of high aeration growth. *A. vinelandii* respiratory membranes were prepared from cells harvested at various stages of $N_2$–mannitol, high aeration growth and assayed for P/2*e* ratios as described in MATERIALS AND METHODS. △—△, P/O NADH; □—□, P/O malate (equivalent to P/2*e* at Site II); ○—○, P/2*e* Site I (NADH *minus* malate). The vertical dotted line indicates the turnover point from logarithmic into non-logarithmic, oxygen-limited growth.

Fig. 3. Dehydrogenase/terminal pathway activity ratios of isolated respiratory membranes as a function of high aeration growth. *A. vinelandii* respiratory membranes were prepared from cells harvested at various stages of $N_2$–mannitol, high aeration growth and assayed for oxygen uptake in the absence of ADP. Typical oxidase activities (μgatoms O/min per mg protein) with NADH and malate, respectively, were 1.10 and 0.36 (at early logarithmic growth), 1.42 and 0.98 (at the turnover into oxygen-limited, non-logarithmic growth) and 1.62 and 2.06 (at late oxygen-limited growth); *i.e.* the absolute rates showed an upward trend over the growth curve. Dehydrogenase/terminal pathway activity ratios were calculated as described in MATERIALS AND METHODS. Substrates, NADH (●—●), malate (■—■) and the combined substrates NADH *plus* malate (▲—▲). The vertical dotted line indicates the turnover point from logarithmic into oxygen-limited, non-logarithmic growth.

The extent to which the primary dehydrogenases limited electron transfer rates from substrate to oxygen also varied considerably, but reproducibly, over the growth curve (Fig. 3). The NADH dehydrogenase/terminal pathway activity ratio was highest during early logarithmic growth and steadily decreased thereafter, whereas the ratio with malate as substrate followed the opposite pattern and was highest during late non-logarithmic growth. With the combined substrates NADH *plus* malate oxygen uptake was no longer limited by the dehydrogenases and the dehydro-

genase/terminal pathway activity ratio increased from 1.15 to 1.50 over the growth curve. Unfortunately the NADH–juglone reductase/NADH dehydrogenase activity ratio was variable over the range 2.0–4.5 and no clear trends of the type shown in Fig. 3 were observed as a function of growth.

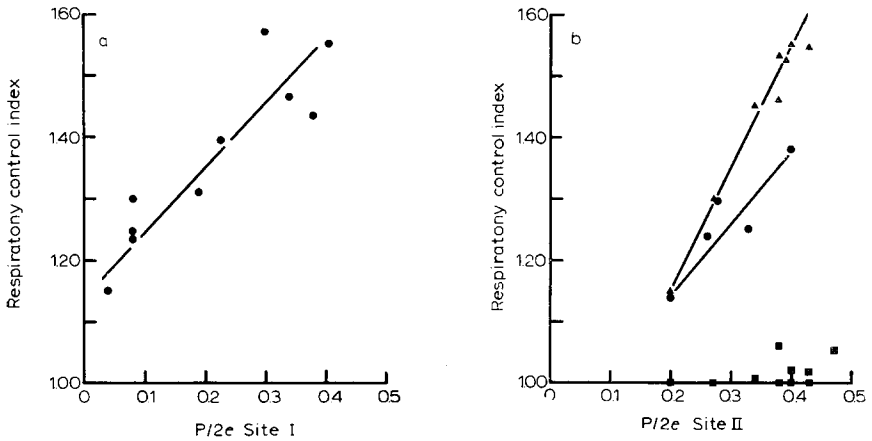


Fig. 4. Respiratory control as a function of P/2e ratios at Sites I and II. *A. vinelandii* respiratory membranes were prepared from cells harvested over selected narrow ranges of the growth curve and assayed for oxygen uptake, respiratory control and P/2e ratios as described in MATERIALS AND METHODS. Electron transfer activity ratios were calculated as described earlier. Each point represents a separate membrane preparation. a. P/2e Site I variable as indicated. Substrate, NADH (●—●); P/2e Site II in the narrow range 0.33–0.43 and NADH–juglone reductase/NADH dehydrogenase activity ratios in the narrow range 2.5–3.5. b. P/2e Site II variable as indicated. Substrate, NADH (●—●); P/2e Site I $\leqq$ 0.08 and NADH dehydrogenase/terminal pathway activity ratios in the narrow range 0.88–0.92. Substrate, malate (■—■); malate dehydrogenase/terminal pathway activity ratio $<$0.80. Combined substrate, NADH *plus* malate (▲—▲); NADH *plus* malate dehydrogenase/terminal pathway activity ratio 1.15–1.40.

In spite of very careful attempts to prepare each batch of respiratory membranes under identical conditions, the absolute rates of oxygen uptake and phosphate esterification expressed on a protein basis were only poorly reproducible (a variation of up to 50% between expectedly similar preparations was often observed) presumably due to the presence of varying amounts of non-respiratory protein. On the other hand most relative parameters (P/2e ratios and dehydrogenase/terminal pathway activity ratios) showed good reproducibility between expectedly similar preparations and exhibited smooth changes as a function of growth (see Figs. 2,3), and for this reason we chose to examine the effects of these parameters and not the absolute rates on the efficiency of respiratory control.

By isolating respiratory membranes from cells harvested over carefully selected narrow ranges of the high aeration growth curve (*e.g.* $A_{680\,nm}$ = 0.25–0.47) we obtained membrane preparations in which one respiratory chain parameter (*e.g.* P/2e at Site II) varied markedly but was accompanied by only relatively small variations in the others (*e.g.* P/2e at Site I and the dehydrogenase/terminal pathway activity ratios). In this way we were able to investigate the gross effects (Figs. 4,5) on respiratory control indices both of the efficiency of energy coupling at Sites I and II (assayed as P/2e ratios) and of the extent of rate-limiting electron transfer at those sites (expressed as relative rates of electron transfer over different segments of the respiratory chain).

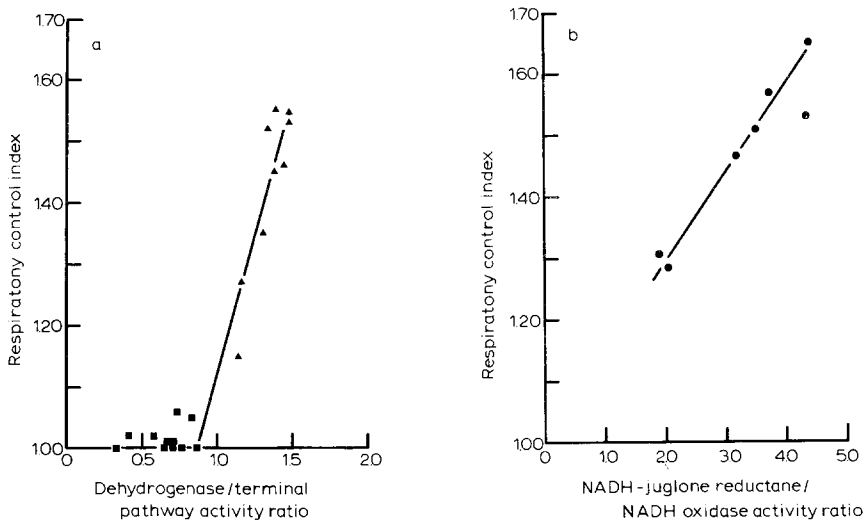


Fig. 5. Respiratory control indices as a function of the extent of rate limitation in electron transfer at phosphorylation Sites I and II. Experimental details as for Fig. 4. a. Extent of rate limitation in electron transfer at Site II (malate dehydrogenase/terminal pathway and NADH *plus* malate/terminal pathway activity ratios) variable as indicated; P/2*e* Site II 0.30–0.43. Substrate, malate (■—■). Combined substrate, NADH *plus* malate (▲—▲). b. Extent of rate limitation in electron transfer at Site I (NADH–juglone reductase/NADH dehydrogenase activity ratio) variable as indicated; P/2*e* Site I 0.30–0.45. Substrate, NADH (●—●).

With NADH as substrate (Fig. 4a) the respiratory control index was found to vary linearly as a function of Site I energy coupling over the P/2*e* range 0.08–0.40. On the other hand in membranes which exhibited very low energy coupling at Site I (P/2*e* < 0.10) but very high dehydrogenase/terminal pathway activity ratios (>0.90) the respiratory control index with NADH was proportional to the efficiency of energy coupling at Site II. A steeper plot was observed with a mixture of NADH *plus* malate, presumably due to the higher NADH *plus* malate dehydrogenase/terminal pathway activity ratios (>1.15). With malate alone as substrate the respiratory control index remained below 1.06 over the entire Site II P/2*e* range, presumably due to the low malate dehydrogenase/terminal pathway activity ratios (<0.80; see Fig. 4b).

In membranes with relatively high energy coupling at Site II (P/2*e* > 0.30; Fig. 5a) the respiratory control index with the combined substrates NADH *plus* malate was found to increase linearly as a function of the increasing NADH *plus* malate dehydrogenase/terminal pathway activity ratios. No significant respiratory control was observed with malate alone since the malate dehydrogenase/terminal pathway activity ratios never exceeded 0.85 in this series of particles (*i.e.* malate dehydrogenase was rate limiting for malate oxidase in all preparations).

The ability of membranes with relatively high energy coupling at Site I (P/2*e* > 0.30; Fig. 5b) to exhibit respiratory control with NADH was determined by the NADH–juglone reductase/NADH dehydrogenase activity ratio; respiratory control increased linearly with increasing values for this ratio.

10 μM KCN, sufficient to completely inhibit the minor terminal pathway, did not alter the observed respiratory control indices, thus eliminating involvement of Site III.

## CONCLUSIONS

The results described in this paper extend earlier reports[11,12] that partly coupled respiratory membranes of the nitrogen-fixing aerobe *A. vinelandii* exhibit respiratory control.

In partly coupled respiratory membranes of this type the steady state level of phosphorylation intermediates accumulated in the absence of phosphate acceptor is presumably low and, in consequence, the inhibition of respiration is also low. The dissipation of these intermediates following the addition of ADP therefore causes only a relatively poor stimulation of electron transfer. It is obvious that this stimulation, expressed as the respiratory control index (+ADP/—ADP) is detectable only when it results in an increased rate of oxygen uptake. For this to occur an energy coupling site must be coincident with the rate-limiting step of respiration[12].

Respiratory control is therefore observed with NADH (since Site I is coincident with the rate-limiting step in NADH dehydrogenase) and with the combined substrates NADH *plus* malate (since the rate-limiting step of electron transfer is now coincident with Site II), but not with malate alone (since no phosphorylation site is associated with the rate-limiting malate dehydrogenase). The ability of 2-*n*-alkyl 4-hydroxyquinoline *N*-oxide to induce substantial respiratory control with malate (see refs. 12, 25, 26) by virtue of its ability to inhibit electron transfer in the region of Site II and thus to shift the rate-limiting step of electron transfer from malate dehydrogenase to the region of Site II, fully supports this conclusion.

The results described in this paper clearly show that once this first criterion is satisfied the respiratory control index is proportional to the extent of the rate limitation of electron transfer at the controlling site and also to the efficiency of energy coupling at that site. Thus the highest observed respiratory control index with NADH (respiratory control index = 1.65) results from a combination of particularly severe rate limitation of electron transfer at Site I (seen as an NADH–juglone/NADH dehydrogenase activity ratio of 4.40) and of high energy coupling at that site ($P/2e$ = 0.35). Similarly the highest observed respiratory control index with the combined substrates NADH *plus* malate (respiratory control index = 1.55) results from a combination of severe rate limitation of electron transfer at Site II (seen as an NADH *plus* malate dehydrogenase/terminal pathway activity ratio of 1.47) and of high energy coupling at that site ($P/2e$ = 0.43).

The lack of respiratory control with malate under normal, uninhibited conditions provides yet another example of a bacterial membrane preparation exhibiting considerable energy coupling without concomitant respiratory control (see ref. 14). It is likely that at least some of these failures may stem from the inability of the preparations involved to satisfy the criterion of coincidence between sites of rate-limiting electron transfer and of energy conservation.

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