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OXIDATIVE PHOSPHORYLATION IN *AZOTOBACTER VINELANDII* PARTICLES

PHOSPHORYLATION SITES AND RESPIRATORY CONTROL

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SUMMARY

1. Oxidative phosphorylation by a particulate preparation from *Azotobacter vinelandii* was measured with succinate, malate or NADH as substrate. The P/NADH values were higher than the P/succinate and the P/malate ratios. The anaerobic reduction of ubiquinone-1 by NADH was also coupled to phosphorylation. It is concluded that there are at least two phosphorylation sites in Azotobacter particles.

2. The P/NADH ratio is not influenced by low concentrations of cyanide.

3. The NADH oxidase activity of phosphorylating particles is stimulated 20–40% by ADP in the presence of phosphate and $MgCl_2$. This stimulation is not found in non-phosphorylating particles.

4. The K_m for ADP in the phosphorylating particles is 10–14 μM, depending on the salt concentration.

The respiratory chain in *Azotobacter vinelandii* particles is characterized by a very high oxidation rate, a low phosphorylation yield and a low sensitivity towards many uncouplers[1-3]. The low P:O ratios might be explained by the presence of a phosphorylating and a non-phosphorylating pathway, to the existence of only one phosphorylation site between NADH and oxygen or to the breakdown of a "high-energy intermediate" or "high-energy state".

JONES AND REDFEARN[4] proposed a scheme for a branched respiratory chain, one containing cytochrome b_1 being relatively insensitive towards cyanide, and the other containing cytochrome c_4 being completely inhibited by 10 μM cyanide. Since this concentration of cyanide inhibits the NADH and succinate oxidation only slightly, it was concluded that the major pathway is *via* cytochrome b_1. In our hands, the P/NADH ratio is not altered by the presence of 100 μM cyanide (Table I), suggesting that the pathway *via* cytochrome b_1 is phosphorylating, although the possibility of a third pathway is not excluded.

P:O ratios are consistently higher with NADH than with succinate or malate as substrate (Table II). This points to the existence of at least two phosphorylation

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sites, one between NADH and the point where the electrons from succinate enter the chain (presumably ubiquinone) and one or more after this point. The fact that the P/malate ratios are similar to the P/succinate is in agreement with the observation of JONES AND REDFEARN[3] that malate oxidation in Azotobacter particles is not NAD linked. The existence of Site-I phosphorylation was confirmed by the finding that the anaerobic reduction of ubiquinone-1 by NADH is also accompanied by phosphorylation (Table III).

As mentioned by KOTELNIKOVA AND IVANOVA[5] the NADH oxidase activity of phosphorylating particles is stimulated 10–40% by the presence of 0.1 mM ADP (Table IV). This stimulation was not found with non-phosphorylating particles, when $MgCl_2$ was omitted, or when ADP was replaced by AMP, ATP or GDP. With limiting amounts of ADP the NADH oxidase activity is lowered again to the original value when all the ADP is phosphorylated. Non-phosphorylating particles incubated for

TABLE I

INFLUENCE OF KCN ON P/NADH RATIOS WITH AZOTOBACTER PARTICLES

Reaction medium 30 mM phosphate (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1–1.5 mM ADP, 10 mM glucose, 1–2 units hexokinase per ml, 0.6 mM NADH, 0.15–0.25 mg particles per ml. Reaction for 2–5 min at room temperature (20–23°). P/NADH ratio is expressed as μmoles glucose 6-phosphate formed per μmole of substrate oxidized

Expt No	*KCN (mM)*	*P/NADH*
1	0	0.32
	0.1	0.30
	0.25	0.27
	0.5	0.14
2	0	0.45
	0.05	0.45
	0.1	0.43

TABLE II

COMPARISON OF THE P/NADH, P/MALATE AND P/SUCCINATE RATIOS

Reaction medium as described in Table I. Substrate concentrations: 0.6 mM NADH, 0.5 mM malate or 0.5 mM succinate. The reaction was stopped after 10 min at room temperature (20–23°). NADH and malate were completely oxidized in that period. P/substrate ratios are expressed as μmoles glucose 6-phosphate formed per μmole of substrate oxidized

Expt No	*P/NADH*	*P/malate*	*P/succinate*
1	0.38	—	0.21
2	0.39	—	0.25
3	0.34	—	0.15
4	0.35	—	0.21
5	0.30	0.25	0.25
6	0.39	0.28	0.28
7	—	0.25	0.19
8	—	0.24	0.22

TABLE III

P/2*e* VALUES FOR THE ANAEROBIC REDUCTION OF UBIQUINONE-1 BY NADH

Reaction medium as in Table I, with in addition 0 4–0.5 μmole ubiquinone-1, 0 27 mM NADH, and 0 9–1 mg particles Reaction volume, 3 ml The reaction was carried out in Thunberg cells in a nitrogen atmosphere The reaction was followed at 340 nm till NADH was no longer oxidized NAD^+ and glucose 6-phosphate were determined enzymically in the deproteinized mixture Both values were corrected for a blank run without ubiquinone-1 The P/2*e* ratios are expressed as μmoles glucose 6-phosphate per μmole of NAD^+ formed

Expt No	*NAD^+ (μmole)*	*Glucose 6-phosphate (μmole)*	*P/2e*
1	0 51	0 095	0 19
2	0 46	0 099	0 22
3	0 52	0 115	0 22

TABLE IV

EFFECT OF ADP ON NADH, MALATE AND SUCCINATE OXIDATION BY PHOSPHORYLATING PARTICLES

Oxidase activities were measured spectrophotometrically at 340 nm in Expts 1–3 and with a Clark oxygen electrode in Expts 4 and 5. Reaction medium 30 mM phosphate (pH 7 6), 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM ADP where mentioned, 0.1–0.12 mM NADH (Expts. 1–3), 0 7 mM NADH or 6 mM succinate or malate (Expts 4 and 5). Particles, 0 5–1 μg/ml (Expts. 1–3) or 15–80 μg/ml (Expts 4 and 5) Temp , 23° (Expts 1–3) or 25° (Expts 4 and 5).

Expt No.	*Substrate*	*Oxidase activity (μatoms O per min per mg protein)*	
		−ADP	*+ADP*
1	NADH	6 1	7.1
2	NADH	6.7	8 4
3	NADH	6 8	9 7
4	NADH	6 1	7.7
	Succinate	0 77	0 76
5	NADH	8 3	10 0
	Malate	6.1	6 3
	Malate + NADH	11 4	13 6

TABLE V

RELATION BETWEEN PHOSPHORYLATING CAPACITY OF THE PARTICLES AND STIMULATION OF NADH OXIDATION BY ADP

Reaction medium as in Table IV In the first experiment non-phosphorylating particles (P^-) were incubated for 45 min with a soluble factor of oxidative phosphorylation (S) at a concentration of 0 9 mg P^- and 0 45 mg S per ml in the presence of 0 1 M KCl. The incubation mixture was then diluted 1 100 with 0 1 M KCl and the NADH oxidase was measured at 340 nm with 0 1 ml of the diluted enzyme In the second experiment the factor was added to the particles after dilution and the reaction was started immediately Oxidase activities are expressed per mg particle protein

Expt No	*Fraction tested*	*NADH oxidized (μmoles/min per mg protein)*		*Ratio +ADP/−ADP*	*P/NADH*
		−ADP	*+ADP*		
1	(P^- + S)	5 6	7.4	1 32	0 33
2	P^- + S	6 7	6 7	1.00	0.02

TABLE VI

INHIBITION OF NADH OXIDASE BY 2-HEPTYL-4-HYDROXYQUINOLINE-*N*-OXIDE IN PRESENCE AND ABSENCE OF ADP

NADH oxidase activity was measured as described in Table IV, Expts 1–3

Inhibitor (*μM*)	*NADH oxidase* (*μmoles NADH per min per mg protein*)	
	− ADP	*+ ADP*
—	6 7	8 4
0 1	6 7	8 4
0 5	6 3	8 1
1.0	5 7	7 6
5 0	3 4	4 8

45 min with a soluble phosphorylation factor in the presence of 0.1 M KCl regain the capacity to phosphorylate[2,6]. The stimulation of the NADH oxidase by ADP is also restored (Table V). When the factor was added without preincubation the particles did not become phosphorylating, nor was stimulation by ADP restored. These experiments indicate that the stimulation by ADP is connected with oxidative phosphorylation. Stimulation by ADP and phosphate has also been found in phosphorylating particles from *Alcaligenes faecalis*[7]. No stimulation by ADP has been demonstrated with mammalian submitochondrial particles.

The stimulation of the NADH oxidation by ADP would be explained if both a tightly coupled phosphorylating pathway and a non-phosphorylating pathway are present, or if the breakdown of a "high-energy" intermediate or state is the limiting factor for electron transport in the absence of ADP. The first possibility is not supported by the lack of stimulation by ADP of the malate or succinate oxidation, unless we assume that the observed respiratory control with NADH oxidation is connected

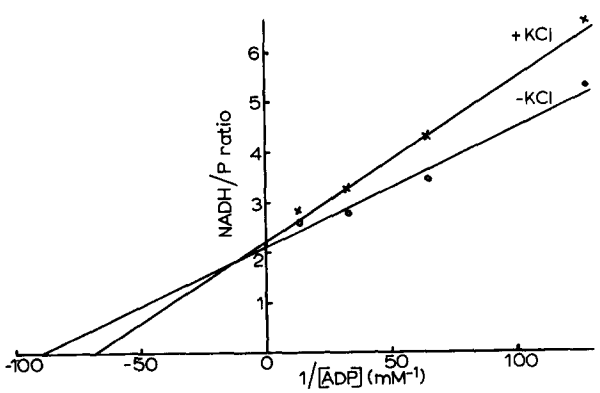

Fig 1 Double-reciprocal plot of the rate of phosphorylation of ADP (assumed to be proportional to the P/NADH ratio) *versus* the concentration of ADP O—O, normal reaction mixture of oxidative phosphorylation (see Table I) containing 49 mM Na^+ and 9 mM K^+ The hexokinase concentration was increased to 4 units/ml; particles, 0 05 mg/ml ×—×, same reaction mixture with an additional 50 mM KCl

with Site I only. The second possibility is not supported by the fact that the stimulation of NADH oxidation by ADP is still present when the oxidation is inhibited by 2-heptyl-4-hydroxyquinoline-*N*-oxide (Table VI). At the concentrations used this inhibition does not influence the P/NADH ratios.

The K_m for ADP was estimated by measuring P/NADH values with varying ADP concentrations. The assumption was made that the P/NADH ratio is proportional to the rate of phosphorylation of ADP to ATP. According to PAPA *et al.*[8], the K_m for ADP in submitochondrial particles is dependent on the concentration of monovalent cations that inhibit competitively with respect to ADP. They found a value of 40 μM in the presence of 66 mM $Tris^+$ and 80 μM in the presence of 66 mM $Tris^+$ *plus* 30 mM K^+. In Azotobacter particles KCl is also a competitive inhibitor for ADP (Fig. 1). The K_m observed was 11 μM in the presence of 58 mM ($K^+ + Na^+$) and 14 μM in the presence of an additional 50 mM KCl. The lower K_m for ADP in Azotobacter particles may be related to the fact that the influence of KCl is less.

EXPERIMENTAL

Azotobacter vinelandii was grown in 16-l or 100-l batches as described elsewhere[2]. Occasionally extra iron (total concentration 8 mg/l) and sucrose (up to 6 %) was added[9]. The preparation of the cell-free extract, the phosphorylating and non-phosphylating particles and the soluble factor of oxidative phosphorylation as well as the measurement of oxidative phosphorylation with NADH as a substrate are described in ref. 2. The amount of malate oxidized was routinely determined by measuring the oxaloacetate *plus* pyruvate formed, with NADH and lactate and malate dehydrogenase. In control experiments these values agree with the oxygen uptake, measured with an Oxygraph, and the malate disappearance estimated by measuring before and after the reaction the malate concentration in the deproteinized reaction mixture with malate dehydrogenase and NAD^+.

For the determination of the P/succinate ratio, the succinate concentration was measured before and after the reaction according to the method of MASSEY[10] as modified by VEEGER AND ZEYLEMAKER[11]. Since the phosphorylating particles may contain a trace of fumarate hydratase[1], the formation of pyruvate *plus* oxaloacetate was also measured and the values of the glucose 6-phosphate formed were corrected for the glucose 6-phosphate corresponding to malate oxidation. This correction was small. In the experiments given in Tables II and III the reaction was stopped by addition of $HClO_4$ and analyses were done in the neutralized supernatant after removal of the perchlorate as the potassium salt.

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