OXIDATIVE PHOSPHORYLATION IN INTACT *AZOTOBACTER VINELANDII*

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1. Introduction

Recently Hempfling [1] described a method to determine directly the efficiency of oxidative phosphorylation in intact cells of *Escherichia coli*. In the nitrogen fixing microorganism *Azotobacter vinelandii* the P/O ratios in cell-free extracts are low [2, 3]. Furthermore, uncertainty exists about the number of phosphorylation sites [3] and the possible participation of a non-phosphorylating pathway [2]. Although Knowles and Smith [4] estimated that the P/O ratio is about 2, we decided to measure directly the efficiency of the oxidative phosphorylation in intact Azotobacter cells by Hempfling's method [1]. Our results show that the oxidation of one molecule of NADH is coupled to the formation of three molecules of ATP, independent of the presence of a nitrogen fixing system.

Also, direct measurement of the P/O ratio, using limiting amounts of oxygen, indicate a complete coupling between oxidation and phosphorylation in intact Azotobacter cells.

2. Methods

A. vinelandii (strain ATCC 478) was grown and harvested as described elsewhere [5]. The mutant 84R1 was grown in a medium containing 0.2% KNO_3 [6]. To measure oxidative phosphorylation, cells (in 25 mM potassium phosphate, pH 7.6, 5–10 mg protein/ml) that had been anaerobic for 30 min under nitrogen or argon, were rapidly mixed with phosphate buffer (25 mM, pH 7.6), saturated with air. After 0.3–3 sec 0.4 vol of 40% (w/v) perchloric acid was added to fix the endogenous adenine nucleotides and NAD^+. After neutralization, NAD^+, AMP, ADP and ATP were measured fluorimetrically according to Williamson and Corkey [7]. Oxygen uptake was measured with a Clark-type electrode. Protein was determined as described by Gornall et al. [8].

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3. Results

When using changes in levels of adenine nucleotides and NAD^+ as a measure for oxidative phosphorylation in bacteria, one has to be sure that there is no turnover of either NAD or adenine nucleotides. Fig. 1 shows the levels of endogenous NAD^+ and ATP in sucrose-grown cells under aerobic and anaerobic conditions.

Fig. 1A shows that under anaerobic conditions the endogenous NAD^+ is rapidly reduced and the ATP level sharply decreases. In fig. 1B the cells are depleted of endogenous substrates by aeration at 25° for 60 min. In these depleted cells both reduction of NAD^+ and disappearance of ATP are much slower. Because of these differences, we have measured oxidative phosphorylation under both conditions. Furthermore, we have measured oxidative phosphorylation in cells grown on either sucrose or succinate as a carbon source, and also in the mutant 84R1 that lacks the nitrogen fixing system [6].

Fig. 2 shows the changes in the levels of the adenine nucleotides and NAD^+ on adding oxygen to anaerobic cells. Table 1 gives the calculated corresponding

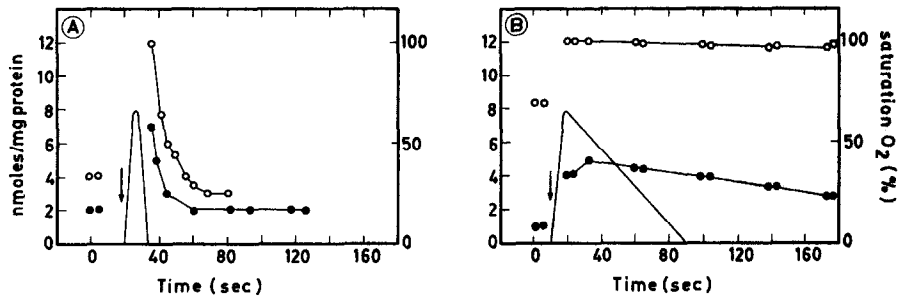

Fig. 1. Levels of ATP and NAD^+ in intact cells of *A. vinelandii* under aerobic conditions. Cells were suspended in a medium containing 25 mM phosphate buffer (pH 7.6) and 100 μg catalase (Boehringer), at a protein concentration of 10 mg/ml. Temp, 25°. Samples were taken at different times and analysed as described in Methods. At the time indicated by the arrow, oxygen was supplied to the anaerobic solution by adding H_2O_2. (A) sucrose cells, not depleted of endogenous substrates. (B) sucrose cells, depleted of endogenous substrates. o—o, NAD^+; •—•, ATP; ——, O_2.

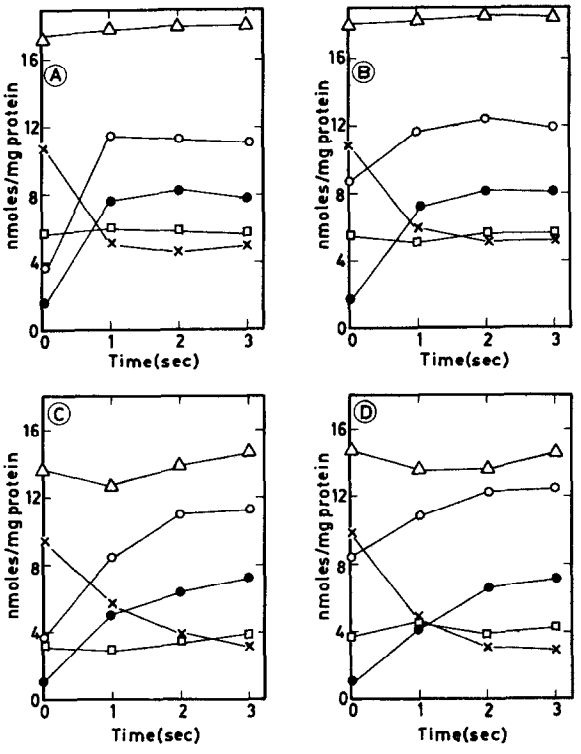

Fig. 2. Changes in the levels of adenine nucleotides and NAD^+ during oxidative phosphorylation in intact *A. vinelandii*. The reaction was carried out as described in Methods. Temp, 25°. (A) sucrose cells. (B) sucrose cells, depleted of endogenous substrate. (C) mutant 84R1. (D) mutant 84R1, depleted of endogenous substrates. o—o, NAD^+; •—•, ATP; □—□, ADP; x—x, AMP; △—△, sum of adenine nucleotides.

P/NADH ratios. The numbers are the mean of at least 3 experiments. Although we use argon to make the cell suspension anaerobic, nitrogen gives the same result.

From table 1 it can be seen that in non-aerated succinate-grown cells the extent of NADH oxidation is very variable. This is probably caused by the fact that substrates other than NADH are oxidized, presumably intermediates of the Krebs cycle. This is confirmed by the observation that depleted succinate-grown cells, incubated with succinate, give the same results as non-depleted cells.

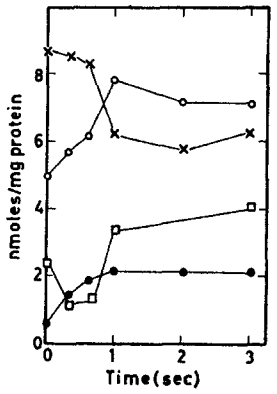

Fig. 3. Oxidative phosphorylation in intact *A. vinelandii* at 1°. Sucrose cells, not aerated, were suspended at 25°, and made anaerobic. After 25 min, the temp was lowered to 1°. At this temperature, the reaction was carried out as described further in Methods. o—o, NAD^+; •—•, ATP; □—□, ADP; x—x, AMP.

Table 1
Changes in adenine nucleotides and NAD^+ during oxidative phosphorylation in intact *Azotobacter vinelandii*.

Cells (grown on)	[ΔATP + (−ΔAMP)] (nmoles/mg protein)	ΔNAD⁺ (nmoles/mg protein)	P/NADH ratio
Sucrose−	11.9 (11.5–12.1)	8.1 (7.9–8.2)	1.5
Sucrose+	9.8 (9.5–10.6)	3.0 (2.9–3.1)	3.3
Succinate−	10.2 (9.9–10.3)	1.8–8.3	(5.7–1.2)
Succinate+	8.9 (8.9– 9.1)	3.1 (2.9–3.3)	2.9
84R1, sucrose−	10.3 (10.0–10.5)	7.0 (6.8–7.3)	1.5
84R1, sucrose+	11.8 (11.7–11.9)	3.8 (3.8)	3.1

Reactions were carried out as described in Methods. AMP, ATP and NAD^+ were measured in the neutralized acid extracts. The values are the mean of at least 3 experiments. The P/NADH ratio was calculated according to Hempfling [1]. Temperature, 25°. +: depleted of endogenous substrates; −: not depleted. Extreme values are given in brackets.

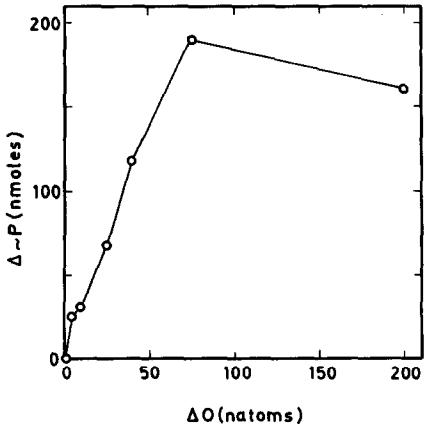

Fig. 4. Relation between oxygen consumption and ATP formation by *A. vinelandii*. To cells, suspended at 25° under argon, were added aliquots of aerobic phosphate buffer. The ATP formed after 1 sec was measured as described in Methods.

Finally, NADP does not contribute significantly, being less than 10% of the NAD level.

Fig. 3 shows an experiment at 1°. The rise in NAD^+ level is kinetically paralleled by an increase in esterified phosphate. Thus, a rapid burst in NADH oxidation, followed by a slower ATP formation coupled to turnover of the NAD, is excluded (see also the experiments in figs. 2C and 2D).

Finally, we have measured the actual P/O ratio by adding limiting amounts of oxygen to an anaerobic Azotobacter suspension. As can be seen in fig. 4, increasing amounts of oxygen lead to increasing amounts of esterified phosphate up to a certain maximum. The slope of the plot indicates that consumption of 1 atom of oxygen is accompanied by the synthesis of nearly 3 molecules of ATP.

4. Discussion

Although cell-free extracts of heterotrophic bacteria generally give a low P/O ratio with different substrates, there are now several indications that these low values must be artificial, arising from the damage during preparation and comparable with results obtained with sub-mitochondrial particles. Direct measurements of oxidative phosphorylation in *E. coli* by Hempfling [1], molar growth yield studies in several bacteria [9] and the H^+/O ratio as measured by Scholes and Mitchell [10] all indicate that oxidation and phosphorylation are completely coupled in intact bacteria.

Since in *A. vinelandii* there is the possible complicating factor of a nitrogen fixing system, we thought it interesting to measure the efficiency in intact cells of this organism. From our results it is clear that oxidation of one molecule of NADH is coupled to the synthesis of 3 molecules of ATP as indicated by a P/NADH ratio of 2.9–3.3. The finding that the P/NADH ratio is lower in non-depleted cells is probably the result of a balance between the ATP consuming reactions and the turnover of NAD. That it has nothing to do with nitrogen fixation is shown by the fact that anaerobiosis induced by argon or nitrogen gives the same results, and by the fact that similar results are also found in the mutant 84R1, that lacks the nitrogen fixing system.

Although at 25° only the levels of ATP and AMP

change and ADP remains constant, at 1° we see that the ADP level is first lowered and later on increases. According to the explanation of Hempfling [1] this would mean that at 1° the adenylate kinase is relatively less active than the phosphorylating enzymes as compared with 25°. Also from fig. 3 it can be concluded that oxidative phosphorylation is not uncoupled at 1°, in contrast to the suggestion by Hempfling [1].

Acknowledgements

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