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Respiratory Control in *Micrococcus lysodeikticus*

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Abstract

The respiration rate of P_i-deprived cells of *Micrococcus lysodeikticus* is markedly increased by P_i, and returns to the original level following P_i consumption. The stimulation of the respiration was found to be specific for P_i and arsenate. Although succinate and valinomycin enchanced the respiration of both P_i-grown and P_i-deprived cells, only the latter could be further stimulated by P_i. The effect of P_i on the respiration rate was found to be concentration dependent. The control of respiration by P_i is due to its rapid uptake and its subsequent polymerization to polyphosphate via ATP. Both of these processes are coupled to proton influx into the cell, and thus stimulate the proton efflux and the respiration rate.

Key Words: Respiratory control; phosphate transport; polyphosphate; proton-motive force; *Micrococcus lysodeikticus.*

Introduction

Respiratory control has been defined as the stimulation of respiration rate by ADP and P_i, followed by return to the original rate on exhauston of either cofactor (Chance and Baltscheffsky, 1958; Racker, 1976). This phenomenon has been widely demonstrated in mitochondria, but not in bacterial cells, although the processes of respiration and ATP synthesis are similar in both systems (Chance and Williams, 1955a,b; Racker, 1976; Haddock and Jones, 1977; Harold, 1972). Growing *Escherichia coli* cells apparently respire at their maximal capacity, since the $\Delta\bar{\mu}_{H^+}$,[3] generated by respiration, is simultaneously depleted by energy-consuming processes, such as active transport and

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[3]$\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons across the cell membrane; $\Delta\psi$, electrical potential across the cell membrane; RCR, respiratory control ratio = (respiration rate after addition)/(respiration rate before addition).

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ATP synthesis. Thus, only when cell growth is arrested, and the respiration rate reduced, can the respiratory control be detected (Burstein *et al.*, 1979; Tsuchiya and Rosen, 1980). Unlike mitochondria, the respiration of whole bacterial cells is not affected by exogenous ADP, which does not permeate the cell envelope (Lichtenstein *et al.*, 1960). Indeed, respiratory control in intact bacterial cells has been demonstrated only indirectly, using uncouplers of oxidative phosphorylation (Bovell *et al.*, 1963; Scholes and Mitchell, 1970; Burstein *et al.*, 1979; Tsuchiya and Rosen, 1980). The common denominator between the effects of uncouplers and ATP synthesis is the reduction of $\Delta\bar{\mu}_{H^+}$ and the subsequent increase in the rate of proton extrusion by the respiratory chain (Harold, 1972; Mitchell, 1973; Rosen and Kashket, 1978).

An alternative method for studying respiratory control, which circumvents the above-mentioned constraints, is to employ cell homogenates or isolated membrane particles in which a fraction of the membranal ATPase is exposed to the external medium. Yet, in cell-free systems, ADP, or ADP and P_i, exerted only a small stimulation of the respiration (Scocca and Pinchot, 1965, 1968; Revsin and Brodie, 1967; Jones and Hamilton, 1970; Eilerman *et al.*, 1970, 1971; Jones *et al.*, 1971a,b; Burstein *et al.*, 1979).

This paper describes, for the first time, the control of electron flow in the respiratory chain via ATP/ADP in intact bacterial cells in a direct manner. The direct demonstration of respiratory control was possible due to the unique phosphate metabolism of *Micrococcus lysodeikticus*. In this organism, both the P_i uptake system, driven by $\Delta\bar{\mu}_{H^+}$, and polyP synthesis via ATP, are markedly derepressed during P_i starvation (Friedberg and Avigad, 1968, 1970; Friedberg, 1977a,b; Alfasi *et al.*, 1979; Friedberg and Kaback, 1980). This correlation between the phosphate metabolism of *M. lysodeikticus* and its respiratory control is discussed.

Materials and Methods

Micrococcus lysodeikticus ATCC 4698 (Fleming) was grown on P_i-buffered defined medium, composed of salts, amino acids, and vitamins, at 30°C, with shaking, to late logarithmic phase. P_i-deprived cells were obtained by additional growth on P_i-less medium, in which P_i was replaced by triethanolamine-HCl buffer (Friedberg, 1977a).

The assays of P_i and arsenate uptake were carried out in P_i-grown and P_i-deprived cells. The cells were harvested, washed twice, and suspended in 50 mM triethanolamine-HCl buffer, pH 7.8, containing KCl and $MgSO_4$ as indicated in the experiments, at room temperature; the cell suspensions were maintained for about 30 min before the assay. The uptake measurements were initiated by addition of either [^{32}P]-P_i or [^{74}As]-arsenate (usually to a final

concentration of 0.1 mM) to 1 ml of cell suspension (final volume) containing about 0.4 mg cell protein, in a 25-ml flat-bottom flask, to ensure adequate aeration. Samples of 200 μl were withdrawn at 30-sec time intervals, filtered through membrane filters (Schlicher and Schull, 0.45 μm porosity), and washed with 5 ml of the uptake medium. The radioactivity on the filters was measured with a Packard 3330 scintillation spectrometer, and the uptake rate estimated from the slope of radioactivity vs. time, which was linear for at least 2 min (Friedberg, 1977a).

Fractionation of P_i, phosphate esters, and polyphosphate (polyP) was carried out as previously described (Friedberg and Avigad, 1968) and assayed according to Avron (1960).

Oxygen consumption was measured with a Clark type electrode at 30°C (Beechy and Ribbons, 1972). A standard assay system contained 0.5–1.0 mg cell protein per milliliter, in 50 mM triethanolamine-HCl buffer, pH 7.8, 10 mM KCl, 5 mM $MgCl_2$, and other additions, as indicated in the experiments, in a final volume of 5 ml.

Protein was determined by the Biuret method using bovine serum albumin as a standard (Gornell *et al.*, 1949).

Results

Addition of P_i to P_i-deprived cells of *M. lysodeikticus* resulted in a fourfold increase in their respiration rate (Fig. 1 and Table I). When the external phosphate was consumed by the cells, the respiration rate returned to

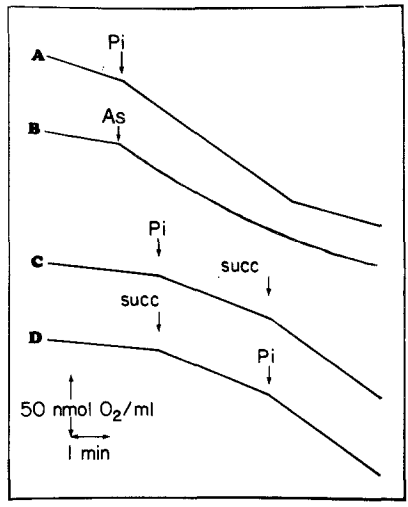

Fig. 1. Effects of P_i, arsenate, and succinate on oxygen consumption rate in P_i-deprived *M. lysodeikticus* cells. Cells were grown, starved for P_i, and prepared for oxygen consumption measurement as described in Materials and Methods. Reaction mixtures contained: (A) 1.5 mg protein per milliliter; P_i was added (arrow) to a final concentration of 80 μM. (B) 0.70 mg protein per milliliter; KH_2AsO_4 (As) was added (arrow) to a final concentration of 4.0 mM; (B, C) 0.38 and 0.33 mg protein per milliliter, respectively, and P_i and succinate (succ) were added (arrows) to final concentrations of 4.0 mM and 10.0 mM, respectively.

Table I. Stimulation of Respiration in P_i-Deprived and P_i-Grown Cells of *M. lysodeikticus*[a]

Additions	Respiratory control ratio	
	P_i-deprived cells	P_i-grown cells
Phosphate	3.9	1.0
Succinate	4.3	6.6
Phosphate + succinate	7.2	6.4
Succinate + phosphate	7.7	6.5
Arsenate	3.5	1.0
Valinomycin	3.9	3.3
Valinomycin + phosphate	5.3	3.3
Phosphate + valinomycin	5.0	3.1

[a]Cell growth, starvation for P_i, and measurements of respiration rates were carried out as described in Materials and Methods. Concentrations used: phosphate, 4 mM; succinate, 10 mM; arsenate, 4 mM; valinomycin, 4 μM. Respiratory control ratio = (respiration rate with addition)/(respiration rate without addition). Second addition was introduced 3 min after the first addition. The endogenous respiration rates (no addition) were 7.6 ($\pm$1.8) and 7.0 ($\pm$1.2) nmol O_2 min^{-1} mg^{-1} of cell protein for P_i-deprived and P_i-grown cells, respectively.

its original value (Fig. 1A). Arsenate, a nonmetabolized analogue of P_i [the uptake of which is mediated by the P_i transport system (Friedberg, 1977a; Alfasi *et al.*, 1979)], exerted a similar increase in the respiration rate (Table I). However, after 3 min the respiration rate gradually decrease, presumably due to the cytotoxicity of arsenate (Fig. 1B). Arsenate inhibits phosphate-dependent synthesis processes, such as polyP synthesis, even in the presence of P_i (Z. Eshhar and I. Friedberg, unpublished data). The stimulation of the respiration rate was specific for P_i and arsenate, as other salts, such as KCl, NaCl, $MgCl_2$, and $MgSO_4$ had no such effect. P_i and arsenate did not affect the respiration rate in P_i-grown cells (Table I).

Addition of succinate, either to P_i-grown or to P_i-deprived cell suspensions, in buffered salt solution, resulted in an increase in the respiration rate (Fig. 1 and Table I). The stimulation of the respiration in P_i-grown cells was about 50% higher than that obtained with P_i-deprived cells. However, only in P_i-deprived cells was an additional stimulation exerted by P_i. The effects of P_i and succinate on the respiration rate in P_i-deprived cells appeared to be mutually independent since an equivalent increase in the RCR exerted by P_i was observed in the presence (increase of 3.8) or absence (increase of 3.9) of succinate. Similarly, the increase of RCR due to succinate addition was approximately the same before (increase of 4.3) or after (increase of 3.3) P_i addition (Fig. 1 and Table I).

Valinomycin, an ionophore for K^+, which effects $\Delta\psi$, exerted an increase of 3- to 4-fold in RCR, both in P_i-grown and P_i-derived cells. However, an additional increase due to P_i addition was obtained only in P_i-deprived cells (Table I).

The magnitude of the stimulation of respiration upon P_i addition to

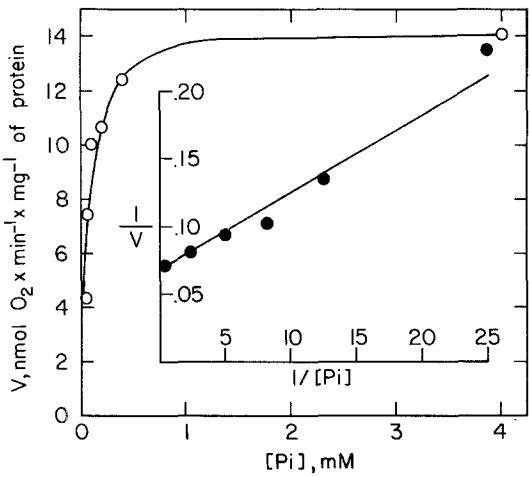

Fig. 2. Increase in the initial rate of respiration (V) as a function of P_i concentration [P_i], in P_i-deprived cells of *M. lysodeikticus*. Growth, starvation for P_i, and measurements of oxygen consumption are as described in Materials and Methods. Inset: a double reciprocal plot (Lineweaver–Burk) of $1/V$ vs. $1/[P_i]$.

P_i-deprived cells was concentration dependent; typical saturation kinetics were observed (Fig. 2). The P_i concentration required for half maximal effect on the respiration rate was estimated to be 85 μM (Fig. 2).

The P:O ratio in P_i-deprived *M. lysodeikticus* cells could be estimated from simultaneous measurements of oxygen consumption and incorporation of P_i into polyP (via ATP). In a typical experiment, a cell suspension of P_i-deprived cells in buffered salt solution was prepared as described in Materials and Methods, containing 1 mg of cell protein per milliliter. [^{32}P]-P_i was added to a final concentration of 0.1 mM. During the first 2 min after P_i addition, 65.6 nmol P_i per milliliter underwent polymerization into trichloroacetic acid-insoluble polymers [mainly polyP (Friedberg and Avigad, 1970)], and 0.9 nmol phosphate per milliliter were found to be soluble phosphate esters (Avron, 1960). The increase in oxygen consumption due to P_i addition was 66 ng-atom O per milliliter. Thus, the P:O ratio was calculated to be 1.0 in P_i-deprived *M. lysodeikticus* cells.

Discussion

The effect of P_i on the respiration rate of *M. lysodeikticus* reflects a mechanism of respiratory control. The direct demonstration of respiratory

control in intact bacterial cells was effected due to the rapid P_i uptake and its subsequent polymerization to polyP, via ATP, in P_i-deprived cells of *M. lysodeikticus* (Kornberg *et al.*, 1956; Friedberg and Avigad, 1968, 1970). The postulated effects of P_i uptake are summarized in Fig. 3. The active transport of P_i, as well as ATP synthesis mediated by membranal ATPase, was shown to be coupled to proton influx (Friedberg, 1977a,b; Alfasi *et al.*, 1979; Friedberg and Kaback, 1980; Huberman and Salton, 1979). ATP is continuously synthesized, as both P_i and ADP are readily available, by the processes of active transport and polyP synthesis, respectively. The continuous proton influx releases the back pressure on the respiratory chain, resulting in an increase in proton efflux and oxygen consumption (Scholes and Mitchell, 1970). Upon exhauston of external P_i, both P_i uptake and polyP synthesis are arrested, and the respiration rate decreases to its original value.

The increase in oxygen consumption elicited by valinomycin is in agreement with the hypothesis that the stimulation of respiration observed here is due to reduction of $\Delta\bar{\mu}_{H^+}$. Valinomycin was shown to decrease $\Delta\bar{\mu}_{H^+}$ in *M. lysodeikticus* (Friedberg, 1977b; Friedberg and Kaback, 1980). Stimulation of respiration elicited by ionophores was reported in additional bacterial strains (Bovell *et al.*, 1963; Scholes and Mitchell, 1970; Burstein *et al.*, 1979;

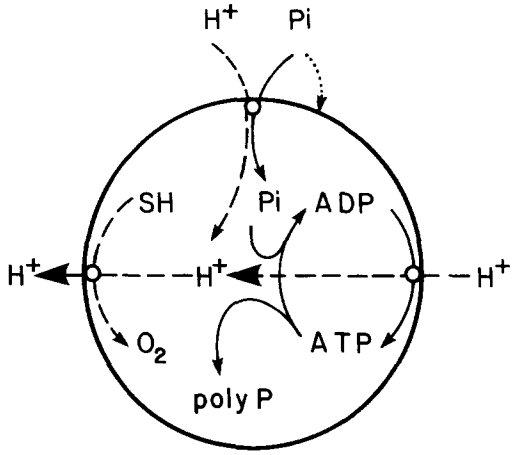

Fig. 3. Schematic presentation of the release of respiratory control by P_i in P_i-deprived *M. lysodeikticus* cells. P_i transport is coupled to proton influx. P_i polymerization to polyP via ATP results in proton influx by the membranal ATPase. Both processes decrease $\Delta\bar{\mu}_{H^+}$ and thus increase proton extrusion by the respiratory chain and oxygen consumption. P_i also affects the membrane externally. Symbols: SH, oxidizable substrate; polyP, polyphosphate.

Tsuchiya and Rosen, 1980). Valinomycin elicited an increase in RCR both in P_i-grown and P_i-deprived cells. However, only in the latter was further stimulation obtained upon P_i addition, indicating an additional dissipation of $\Delta\bar{\mu}_{H^+}$ due to P_i uptake and its metabolism. Under the conditions employed, especially the low external concentration of KCl (10 mM), valinomycin induces K^+ efflux down its concentration gradient, and generation of a diffusion potential across the cell membrane, negative inside. The diffusion potential partially counteracted the dissipation of $\Delta\bar{\mu}_{H^+}$. Thus, the overall potential was indeed reduced, but not abolished (Harold *et al.*, 1975). Then P_i uptake and its subsequent metabolism in P_i-deprived cells elicited an additional dissipation of $\Delta\bar{\mu}_{H^+}$, which was expressed as an additional increase in RCR.

The differences between the P_i concentration required for half-maximal effect on the respiration rate (85 μM) and the K_m for P_i transport (4.3 μM; Friedberg, 1977a) are explained by an additional, exogenous effect of P_i on the integrity of the cell membrane. Thus, proline uptake in membrane vesicles of *M. lysodeikticus* was found to be more efficient when the vesicles were isolated in buffers containing P_i, as compared to P_i-less buffers (I. Friedberg and H.R. Kaback, unpublished observation). In addition, P_i increased the rate of NADH oxidation in particles of *M. lysodeikticus,* and this increase was not affected by ADP (Ishikawa and Lehninger, 1962).

Since most of the P_i taken up to P_i-starved cells is rapidly polymerized to polyP via ATP, we estimated the P:O ratio by simultaneous measurements of polyP synthesis and oxygen consumption. The method is fast and reproducible. and the value of the P:O ratio in intact, P_i-deprived cells of *M. lysodeikticus* was found to be 1. A similar value was reported, employing membrane fragments of *M. lysodeikticus* (Ishikawa and Lehninger, 1962).

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