Sodium-controlled Coupling of Respiration to Energy-linked Functions in *Pseudomonas stutzeri*

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SUMMARY

In the presence of K^+ , Na^+ enhanced respiration and activated cellular motility and transport of K^+ , amino acids and phosphate in *Pseudomonas stutzeri*. Inorganic phosphate taken up was rapidly esterified to nucleoside triphosphates and diphosphates. Respiration and phosphate transport showed cooperative responses to Na^+ which increased with temperature. Respiration was more sensitive to polymyxin B in the presence of Na^+ . Dinitrophenol and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone stimulated respiration when either Na^+ or K^+ was absent, but not in the presence of both cations. Therefore, Na^+ is essential for the cytoplasmic membrane of *P. stutzeri* to perform energy-linked functions coupled to respiration.

INTRODUCTION

Pseudomonas stutzeri is a non-halophilic bacterium requiring Na^+ irrespective of growth conditions (Kodama & Taniguchi, 1976). In the presence of K^+ , respiration was enhanced specifically by Na^+ . The respiration rate of exponentially growing organisms was a linear function of the growth rate when limited by the Na^+ concentration. However, the maximum respiration rate induced by excess Na^+ was independent of the growth rate. One hypothesis that explains these findings is that the cation is essential for coupling of respiration to energy metabolism. If this is valid, cellular functions that require energy would be expected to be Na^+ -dependent.

We have investigated the coupling of respiration to the generation of metabolic energy in the presence of Na^+ (plus K^+).

METHODS

Materials. These were obtained as follows: membrane filters, Sartorius; polyethyleneimine (PEI)-cellulose plates and 'Suprapur' grade NaCl and KCl, Merck; nucleotides, Kyowa Hakko, Tokyo, Japan; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), a gift from Prof. Y. Kagawa (later purchased from Boehringer-Mannheim); [^{32}P]orthophosphoric acid, Japan Atomic Energy Research Institute, Tokyo; L-[G- 3H]glutamic acid, The Radiochemical Centre, Amersham.

Media. Three basal media were used. TLGP medium contained: 100 mM-Tris; 40 mM-DL-lactic acid; 30 mM-L-glutamic acid; 10 mM-phosphoric acid; and 2% (v/v) trace element mixture (Sistrom, 1960). The pH was adjusted with HCl at room temperature (20 to 23 °C) so that the pH would be 7.4 at the desired temperature. TLP and TLG media were derived from TLGP medium by omitting glutamic acid and phosphoric acid respectively.

Micro-organisms. *Pseudomonas stutzeri* (van Niel strain, IAM12097) was maintained on

nutrient agar slopes (Kodama, Shimada & Mori, 1969). It was grown at 30°C in TLGP medium supplemented with 10 mM-KCl and NaCl and growth was measured as previously described (Kodama & Taniguchi, 1976). Exponentially growing cultures (40 to 60 Klett units) were chilled on ice and centrifuged at 5000 g for 10 min. Cells were washed three times with one of the three basal media as specified below.

Uptake of K⁺, glutamate and phosphate. Uptake was measured at 30°C using the following membrane filtration method. The bacterial density in the reaction mixtures was 30 to 50 Klett units.

K⁺. Organisms which had been washed with TLGP medium and kept on ice for 15 h were resuspended in the same medium. After 10 min pre-incubation, KCl (final concentration, 1 mM) was added to start the reaction. Samples (1 ml) were transferred at intervals to membrane filters (MF-45, pore size 0.45 µm) and washed with three 2 ml portions of TLGP medium within 30 s. The filters were dried under an infrared lamp and extracted with 1 ml 0.5 M-HCl at 100°C for 30 min. The extracts were centrifuged for 10 min and the K⁺ concentration in the supernatant was then measured.

Glutamate. The reaction mixture was prepared by diluting the suspension of organisms in TLP medium with the same medium supplemented with chloramphenicol (50 µg ml⁻¹) and 5 mM-KCl. The reaction was started by adding 0.3 mM-[³H]glutamic acid (0.6 µCi ml⁻¹). Samples (0.5 ml) were collected on the membrane filters, washed with TLGP medium and then dried as before. The filter was transferred to a scintillation vial for radioactivity measurement.

Inorganic phosphate. Organisms which had been washed with TLG medium were used. The reaction mixture was composed of this medium supplemented with 0.3 to 1 mM inorganic [³²P]phosphate (PO₄³⁻; 20 to 400 µCi ml⁻¹); 5 mM-KCl was added to start the reaction. Samples (20 to 100 µl) were collected on membrane filters, washed with TLGP medium as described for K⁺ uptake and processed as for the glutamate uptake experiments.

Phosphate uptake was expressed as µmol phosphate (g dry wt)⁻¹. The specific activity of the PO₄³⁻ was calculated from the radioactivity of ³²P and the PO₄³⁻ concentration in the reaction mixtures determined by the method of Fiske & SubbaRow (1925).

Phosphate metabolism. Entry of ³²PO₄³⁻ into the intracellular phosphate pools was determined by extracting the acid-soluble phosphates with formic acid. The washed filters were immediately soaked in 1 ml 0.5 M-formic acid in centrifuge tubes which were agitated for 30 s on a vortex mixer. The tubes were again agitated after standing on ice for 20 min before centrifuging at 1000 g for 10 min to remove acid-insoluble material. Total [³²P]phosphate in the extract was estimated by applying a portion of the supernatant to a disc (1 cm diam.) of Whatman 3MM filter paper which was then dried and counted. The concentration of ³²PO₄³⁻ in the extracts was estimated by the method of Sugino & Miyoshi (1964). Intracellular acid-soluble organic [³²P]phosphate was calculated as the difference between total [³²P]phosphate and ³²PO₄³⁻.

Thin-layer chromatography. The intracellular acid-soluble pool (in 20 µl samples) was resolved by thin-layer chromatography on PEI-cellulose plates (20 × 20 cm) using a modification of the method of Neuhaed, Randerath & Randerath (1965). Mixtures of CMP, CDP, CTP, GMP, GDP, and GTP were used as markers. Chromatograms were developed at 20°C with 0.13 M-LiCl/2 M-formic acid (1:1, v/v) to 15 cm above the origin. Plates were then dried and developed in the same direction with 1 M-LiCl/2 M-formic acid (1:1, v/v) to the distance moved by CDP spots (about 6 cm above the origin). Plates were dried and then examined under incident u.v. light. The surface of each plate was divided into four zones corresponding to compounds migrating faster than CMP (i.e. NAD⁺, NADP⁺ and sugar phosphates), mononucleotides, dinucleotides and trinucleotides. The cellulose powder of

each zone was scraped into a separate vial and the ^{32}P radioactivity was counted. Since PO_4^{3-} co-chromatographed with nucleoside diphosphates, the radioactivity of $^{32}\text{PO}_4^{3-}$ in the total acid-soluble fraction was subtracted from that of ^{32}P in the nucleoside diphosphate zone.

Analytical methods. Respiration was measured with an oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio, U.S.A.) at 30°C unless otherwise stated.

Radioactivity was measured in a Packard Tri-Carb liquid scintillation counter using 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophene (BBOT) in toluene (0.4%, w/v) as scintillator.

The concentrations of Na^+ and K^+ were determined in a Hitachi 204 atomic absorption spectrophotometer.

RESULTS

Na^+ -dependence of energy-linked functions

The effects of Na^+ on transport and motility as measurable energy-linked functions of intact cells were tested. All transport systems are apparently Na^+ -dependent (Fig. 1).

Incubation of organisms under Na^+ - and K^+ -deficient conditions for several hours at 0°C caused partial loss of intracellular K^+ . Growth of such partially K^+ -depleted organisms began after a 30 to 40 min lag period when incubated with Na^+ and K^+ . The K^+ content of the organisms was then restored to the original intracellular level (Fig. 1*a*). However, this net accumulation of the cation did not take place under Na^+ -deficient conditions.

The rate of glutamate uptake by the Na^+ -deficient organisms was initially about 10% of the rate exhibited by those with excess Na^+ and gradually slowed down (Fig. 1*b*). A similar result was obtained for leucine uptake.

Phosphate uptake by the Na^+ -deficient cells initially proceeded at a rate less than 5% of that by organisms with excess Na^+ and stopped after 15 min incubation (Fig. 1*c*). This cannot be ascribed to growth of cells incubated with Na^+ , because the time of incubation (10 min) was short with respect to the culture doubling time (60 min).

Pseudomonas stutzeri is highly motile (Palleroni *et al.*, 1970). Since a low level of energy-generating metabolism is sufficient to support motility of bacteria (Adler & Templeton, 1970), we tested whether cells retained their motility under Na^+ -deficient conditions. Small drops of bacterial suspension with and without added Na^+ were observed by dark-field microscopy. The organisms with Na^+ remained vigorously motile 10 min after preparation of the drop, whereas movement of the Na^+ -deficient organisms was initially sluggish but became faster on adding Na^+ .

Effect of Na^+ on phosphate metabolism

Under the conditions in which the data in Fig. 1*c* were obtained, more than 80% of PO_4^{3-} taken up by the bacteria was in the acid-soluble form. Longer incubation led to increased incorporation into the acid-insoluble form. The distribution of $^{32}\text{PO}_4^{3-}$ into different acid-soluble pools was therefore measured (Fig. 2). There was rapid turnover and synthesis of the nucleotide pool in the presence of Na^+ . Addition of Na^+ elicited rapid PO_4^{3-} incorporation into nucleoside triphosphate and nucleoside diphosphate fractions without appreciable lag, while incorporation into other fractions followed a lag period. During the initial few minutes, about three-quarters of the incorporated PO_4^{3-} went into nucleoside di- and triphosphates.

Fig. 1. Effect of Na⁺ on uptake of (a) K⁺, (b) glutamate, and (c) phosphate: ○, medium supplemented with 5 mM-NaCl; ●, Na⁺-deficient control.

Fig. 2

Fig. 3

Fig. 2. Incorporation of PO_4^{3-} into the intracellular nucleotide pool. PO_4^{3-} uptake was measured in TLG medium plus 5 mM-KCl and 0.1 mM- $^{32}\text{PO}_4^{3-}$ (400 $\mu\text{Ci ml}^{-1}$) at 30°C as described in Methods. The arrow indicates time of addition of 5 mM-NaCl. Nucleoside triphosphates (●), diphosphates (■) and monophosphates (○) and other compounds (△).

Fig. 3. Response to Na⁺ of respiration (●) and phosphate uptake (○) in TLG medium plus 5 mM-KCl and 1 mM- $^{32}\text{PO}_4^{3-}$ (20 $\mu\text{Ci ml}^{-1}$) at 30°C.

Table 1. *Effect of temperature on Hill plot parameters of Na⁺-dependent respiration*

The results were from two independent experiments, one of which gave the results shown in Fig. 4.

Temperature (°C)	Hill coefficient (<i>n</i>)	Na ⁺ concentration (mM) giving half max. activation, [S] _{0.5}
23	1.89, 1.79	0.33, 0.42
30	2.27, 2.40	0.47, 0.48
37	2.62, 3.25	0.75, 0.76

Kinetics of coupling of respiration to phosphate uptake

Respiration and PO₄³⁻ uptake rates were simultaneously enhanced in a sigmoidal fashion with increasing Na⁺ concentration (Fig. 3). However, the respiration rate in the absence of Na⁺ was about a quarter of the maximum rate whereas the corresponding PO₄³⁻ uptake rate was negligible. This suggests that PO₄³⁻ uptake is coupled to respiration only in the presence of Na⁺. The coupling efficiency increases with cation concentration, the maximum efficiency being attained above 2 mM.

Effect of temperature on Na⁺-activated respiration

Growth rate, like respiration rate, is a sigmoidal function of Na⁺ concentration (Kodama & Taniguchi, 1976), suggesting that activation of respiration by Na⁺ is a cooperative phenomenon. The kinetics of Na⁺-activated respiration were therefore studied at different temperatures. At each temperature tested, the respiration rate with excess Na⁺ was three or four times that without added Na⁺ and the curve was sigmoidal (Fig. 4). When Hill plots were made of the Na⁺-dependent respiration rates (the difference between the rates with and without added Na⁺), a straight line with a slope greater than 1 was obtained at each temperature. The line became steeper and shifted towards high Na⁺ concentration as temperature increased (Table 1). Thus, the cooperativity of the response of the respiratory system to Na⁺ was intensified at higher temperatures.

Inhibitor studies

The effects of various inhibitors on respiration were studied under Na⁺-deficient conditions and with excess Na⁺. Inhibition of respiration by polymyxin B was the most remarkable because it was altered by Na⁺ (Fig. 5). Although the curves for inhibition with and without added Na⁺ were similar, the curve with Na⁺ was shifted to lower concentrations of the antibiotic, indicating that in the presence of Na⁺ the respiratory system is more sensitive to polymyxin B. The sensitivity to other effective inhibitors of respiration, such as azide and cyanide, was the same with and without added Na⁺.

Table 2 summarizes the effect of uncouplers of oxidative phosphorylation on respiration under different cation conditions. Low concentrations of FCCP and dinitrophenol (DNP) stimulated respiration in the absence of either Na⁺ or K⁺. In contrast, they had no effect when both cations were present. However, PO₄³⁻ uptake which reflects energy-linked metabolism was strongly inhibited, suggesting that Na⁺ deficiency retards energy-linked functions as well as respiration.

Fig. 4

Fig. 4. Effect of temperature on respiration at different Na^+ concentrations in TLGP medium plus 5 mM-KCl: ●, 37°C; ■, 30°C; ▲, 23°C.

Fig. 5

Fig. 5. Inhibition of respiration by polymyxin B in the presence of 5 mM-NaCl (○) and in the absence of added Na^+ (●). Respiration was measured in TLGP medium plus 5 mM-KCl at 30°C.

Table 2. *Effect of uncouplers on respiration and phosphate uptake*

Respiration and phosphate uptake were measured in TLG medium supplemented with 1 mM- $^{32}\text{PO}_4^{3-}$ (35 $\mu\text{Ci ml}^{-1}$). The cation concentration was 5 mM.

Uncouplers	Ionic conditions		Stimulation of respiration (%)	Inhibition of phosphate uptake (%)
	Na^+	K^+		
FCCP (3 μM)	+	+	0	74
	+	—	54	NT
	—	+	30	NT
DNP (100 μM)	+	+	0	80
	+	—	43	NT

+, Cation added; —, no cation added; NT, not tested.

DISCUSSION

Na^+ elicits multiple effects in cation-deficient *P. stutzeri*. Since these effects can be related to functions of the cytoplasmic membrane which performs energy transduction, we conclude that Na^+ exerts its primary effect on the membrane in such a way that energy stored as the energized state of the membrane can be used to drive ATP synthesis, active transport, cellular motility and other energy-linked processes. The following three lines of evidence support this conclusion.

1. The activation of respiration and phosphate uptake exhibits sigmoidal kinetics with respect to Na^+ concentration. This suggests cooperative behaviour or involvement of a highly organized structure, presumably through multiple interacting Na^+ -binding sites in the cytoplasmic membrane. Furthermore, the response of respiration to Na^+ was temperature-dependent. Conformations of functional proteins and their interactions in the membrane are known to be constrained in part by the viscosity of lipids which are in a fluid state in

membrane structures (Singer & Nicolson, 1972). Alteration of the fluidity of the lipid phase by a temperature change would be expected to modify the interactions between membrane proteins, resulting in altered kinetic characteristics of functions inherent in the membrane.

2. Increase in the sensitivity of respiration to polymyxin B in the presence of Na^+ suggests a conformational change in the cytoplasmic membrane accompanied by energetic activation. The antibiotic affects the permeability barriers of Gram-negative bacteria (Newton, 1956). According to HsuChen & Feingold (1973), it interacts selectively with phosphatidyl ethanolamine and is thought to cause drastic perturbations between the lipid molecules. Thus, the higher sensitivity to polymyxin B in the presence of Na^+ could suggest that the cation-binding to the membrane affects the packing of the lipid constituents so that the target molecules become more accessible to the antibiotic and/or that the lipid phase as a whole is rendered more labile to its disorganizing action.

3. The evidence for the existence of some energized state of the membrane comes from the fact that FCCP and DNP stimulated respiration of the Na^+ -deficient cells which could not perform energy-linked functions. These uncouplers influence several functions of the bacterial membrane, for example stimulation of respiration, active transport, oxidative phosphorylation, energy-dependent transhydrogenase (Harold, 1972) and cellular motility (Larsen *et al.*, 1974). These effects are now thought to be due to the ability of uncouplers to dissipate the energized state of the cytoplasmic membrane. Thus, the low respiration rate of *P. stutzeri* may indicate restriction of respiration resulting from a blockade of the use of energy stored as the energized state of the membrane for energy-linked functions. Perhaps the uncouplers increase respiration by 'short-circuiting' the energized state under the Na^+ - or K^+ -deficient conditions.

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