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THE RELATIONSHIP BETWEEN SUBSTRATE-INDUCED RESPIRATION AND SWELLING IN *AZOTOBACTER VINELANDII*

CHRISTOPHER J. KNOWLES* AND LUCILE SMITH
Department of Biochemistry, Dartmouth Medical School, Hanover, N.H. 03755 (U.S.A.)
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SUMMARY

1. When oxidizable substrates are added to a starved suspension of *Azotobacter vinelandii* osmotically shrunken in 0.2 M KCl, a decrease in absorbance is observed which results from a change in light scattering as the cells increase in volume.

2. The rates of swelling and of respiration following addition of substrate varied exactly in parallel through changes of the concentration of substrate, pH and temperature.

3. Carbonyl cyanide *m*-chlorophenylhydrazone, 2,4-dinitrophenol and azide inhibited the rates of swelling and respiration to similar extents; cyanide inhibited the total swelling and O_2 uptake.

4. The respiration rates remained zero order as the swelling progressed, until very low concentrations of oxygen were reached.

5. About one proton was extruded for each 5 oxygen atoms taken up during respiration, but there was very little change in the K^+ content of the medium.

The data suggest that substrate transport is an energy-requiring process, the energy being supplied by the oxidation of the substrate. The properties of and interrelationships between the two processes are discussed.

INTRODUCTION

The selective transport of substances across the semipermeable membrane of bacteria has an important role in the control of cellular metabolism. This transport appears to be an energy-requiring process for many substances. The main energy-yielding reactions of aerobic bacteria are the oxidative reactions of the respiratory chain system and phosphorylation reactions coupled to them, and these systems are associated with the bacterial cytoplasmic membrane[1]. Thus we have made studies of interrelationships between transport of substrates and respiration and phosphorylation in the obligately aerobic bacteria *Azotobacter vinelandii*. This bacterial species has proved to be useful for this kind of experimentation in several respects: (1) the starved cells shrink as a whole when suspended in proper concentrations of impermeable solutes[2], and swelling follows the addition of oxidizable substrates to starved osmotically contracted cells. This swelling can be easily followed by measuring the

Abbreviation: CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

* Present address: Biological Laboratories, University of Kent, Canterbury, England.

resultant changes in turbidity (light-scattering). (2) These bacteria are stable to washing and starving to reduce the supply of endogenous substrates[2]. (3) They are obligate aerobes which cannot derive energy for growth from fermentative reactions.

METHODS

Growth and preparation of bacteria and cell-free extracts

A. vinelandii (NCIB 8660) was cultured, washed and starved as described in a previous paper[2]. The cells were finally suspended in either 10 mM potassium phosphate buffer or 4 mM Tris–HCl buffer (pH 7.4) and kept on ice for no more than 2 h before initiating experiments.

Cell-free extracts were prepared by exposing concentrated suspensions of cells (14–20 mg dry weight per ml) in the phosphate buffer to sonic oscillation for four 30-sec intervals while the cells were cooled in an ice–salt mixture. After exposure the mixture was centrifuged at 12000 × *g* for 15 min and the top 2/3 of the supernatant suspension removed for use as the broken-cell extract.

The concentration of whole cell suspensions was estimated approximately by turbidity measurements, then accurately by drying cells washed twice with distilled water to constant weight at 90°. Protein was assayed by the biuret method[4] in the presence of 0.2 % sodium deoxycholate.

Measurement of absorbance changes

The changes in absorbance (turbidity) accompanying shrinking and swelling of the bacteria were measured with a Cary recording spectrophotometer, Model 14, as described in the preceding paper[2]. At the start of an experiment, 0.2 ml of a solution containing 0.6 mmole of KCl *plus* any other required addition were added to 2.8 ml of bacteria (0.65 mg dry weight per ml) in phosphate buffer. Then 30 μl of substrate were added from a microsyringe and the mixture rapidly stirred. Substrates were added as their potassium salts, where applicable, and 2,4-dinitrophenol and carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP) were added in methanol (final concentration of methanol was 1 %). The rate of swelling was measured during the interval of several minutes before the O_2 in the solution was exhausted. Changes in cell volume were measured directly as described previously[2].

Measurements of respiration

O_2 uptake was measured polarographically with a Clark-type electrode after addition of 30 μl of 1 M substrate to 3 ml of phosphate buffer containing KCl and other required additions and 1.8 mg of bacteria or a suitable amount of broken-cell extract. When D-mannitol was substrate with the extract, 30 μmoles of NAD^+ were also added.

Measurement of changes of pH and of K^+

Changes of pH were measured with an extended scale pH meter (type PHM26, Radiometer, Copenhagen) connected to a miniature concentric pH electrode. The electrode was placed in a closed vessel containing 3 ml of a suspension of about 3 mg of bacteria in 4 mM Tris–HCl buffer (pH 7.4) *plus* the desired concentration of KCl; the mixture was stirred with a magnetic stirring bar. The reaction was started by

the addition of 30 μl of 1 M substrate, and the pH change recorded in the range of pH between 7.50 and 7.15. The changes in proton content (ΔH^+) were calculated with reference to a calibration curve made by measuring the pH changes following addition of 0–200 μl of 50 mM HCl to 3 ml of the same buffer and KCl.

K^+ uptake was measured using a K^+ electrode (Type 4923-Q10; A.H. Thomas Co., Philadelphia) and a calomel reference electrode (Type 4857-H10, Thomas) connected to the pH meter switched to mV reading. K^+ changes were made under the same conditions as those of the measurements of pH change, except that triple volumes and a larger closed vessel were used. Calibration curves were made by adding varying amounts of KCl to 9 ml of 4 mM Tris–HCl buffer (pH 7.4).

Chemicals

2,4-Dinitrophenol and CCCP were purchased from Calbiochem. The substrates were purchased from Sigma Chemical Co. except for mannitol, which was obtained from Fisher Scientific Co. and was recrystallized from hot water.

RESULTS

Addition of a non-penetrating solute to starved intact cells of *A. vinelandii* in dilute buffer results in a decrease of the volume of the cells due to a contraction of the cytoplasmic membrane *plus* cell wall[2]. This decrease in volume is accompanied by an increase in absorbance (turbidity). Fig. 1 shows that the addition of substrates to cells shrunken in 0.2 M KCl is followed by a gradual decrease in the turbidity, which would indicate an increase in the size of the cells. At the time of O_2 depletion (marked by arrows) there is a small "jump" in the absorbance decrease. On anaerobiosis, swelling either stops or continues at a very low rate. The latter is inhibited if a gentle

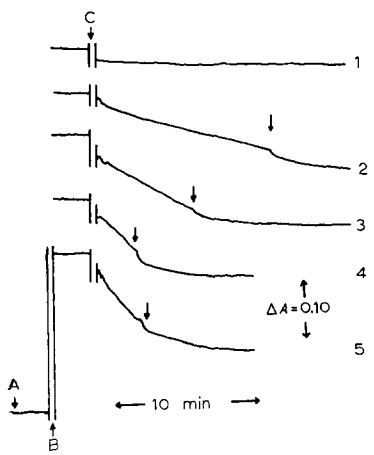

Fig. 1. Substrate induced swelling of osmotically shrunk *A. vinelandii* assayed by the changes in absorbance. 1.8 mg dry wt. of cells were suspended in 2.8 ml 10 mM potassium phosphate buffer (pH 7.4) at 24° to give an initial absorbance of 1.40 at 700 nm (A). At time (B) 0.2 ml of 3 M KCl were added, followed at time (C) by 30 μl of: 1, water; 2, 1 M DL-β-hydroxybutyrate; 3, 1 M pyruvate; 4, 2 M DL-lactate; or 5, 1 M D-mannitol. The parallel lines indicate the intervals required for addition of KCl or substrates. The arrows show the times at which the solutions became anaerobic as measured in parallel experiments with an oxygen electrode.

TABLE I

RATES OF SWELLING AND RESPIRATION OF *A. vinelandii* ON ADDITION OF SUBSTRATE TO CELLS OSMOTICALLY SHRUNK IN 200 mM KCl

0.2-ml samples of 3 M KCl were added to 2.8-ml aliquots of bacteria (0.64 mg dry wt. per ml) in 10 mM potassium phosphate buffer (pH 7.4) and the solutions incubated at 23° for 10 min. 30-μl 1 M substrates were then added and the decreases in absorbance at 700 nm and the oxygen uptake rates were measured as described in METHODS. Substrates were added to give a concentration of 10 mM except for DL-lactate which was present in a 20 mM concentration. Where applicable substrates were present as their potassium salts.

Substrate	*% Decrease in absorbance per min (% ΔA/min)*	*Oxygen uptake (μmoles/min)*	*% ΔA/min* / *μmoles O_2/min*
No substrate	0.050	0.0044	11.4
Acetate	1.03	0.0934	11.0
Ethanol	0.318	0.0238	13.4
DL-β-Hydroxybutyrate	0.375	0.0363	10.3
DL-Lactate	1.59	0.141	11.3
D-Mannitol	1.80	0.161	11.2
Pyruvate	0.840	0.0608	13.8

stream of nitrogen is blown continuously over the cuvette. Thus the low rate seems to result from a slow diffusion of O_2 into the open cuvette.

The changes in absorbance following substrate addition were not due to cell lysis. No decrease in viability was found when the suspensions were tested by the criteria described in the preceding paper[2]. Furthermore, measurements of packed cell volume in hematocrit tubes showed directly that the cells shrank and swelled in parallel with the absorbance changes.

The rate of swelling, as assayed by the decrease in absorbance, was dependent upon the rate of respiration. With a variety of oxidizable substrates the ratio of the rate of swelling to the rate of respiration was relatively constant (Table I). In this experiment O_2 uptake and swelling were measured in parallel experiments with the O_2 electrode and in the spectrophotometer, respectively. The O_2 uptake was observed to remain linear until very low concentrations of O_2 were reached. In later experiments the rates of respiration were estimated from the reciprocals of the time required for the solution to become anaerobic, as indicated by the time when the "jump" in absorbance occurred. The decrease in the osmolarity of the medium due to the loss of substrate could only reach a maximum of 0.01–0.02 out of a total of around 0.44 osM. There was no appreciable decrease in osmolarity resulting from an increased permeability of the cells to K^+ (see below), and similar results were obtained with cells suspended in NaCl, $MgCl_2$ or sucrose. Thus the increase in cellular volume cannot be accounted for by decreased medium osmolarity and must result from an increase in the osmolarity of the bacterial cytoplasm by addition of osmotically active (*i.e.* not membrane-bound) molecules of or derived from the substrate.

Substrate-induced swelling and respiration both reached maximal rates at a substrate concentration of about 10 mM with mannitol; apparent K_m values of 4.2 and 3.5 mM, respectively, were obtained from double reciprocal plots. Similar K_m values were obtained with pyruvate as substrate, but DL-lactate gave values approximately twice as large; this bacterium utilizes only the D(—)-isomer of lactic acid[5].

Therefore the substrate concentration used was 10 mM for all except lactate, where a concentration of 20 mM was employed.

Rates of swelling and of respiration showed parallel changes with variations of pH, with a shallow optimum at pH 7.4. Variation of the reaction temperature between 16° and 32° also produced parallel changes in the rates of swelling and respiration.

The data of Fig. 2 show that CCCP, an uncoupler of oxidative phosphorylation, inhibited respiration and swelling in parallel fashion with lactate as substrate. However, the uncoupler had no effect on the respiration of a cell-free extract of the bacteria in the presence of lactate. Similar observations were made with mannitol as substrate. 2,4-Dinitrophenol and potassium azide also inhibited respiration and swelling of the intact cells in a similar manner; $1 \cdot 10^{-4}$ M 2,4-dinitrophenol caused 50 % inhibition of respiration and swelling and 10 mM azide gave 50 % inhibition. The respiration of cell-free extracts was unaffected by 2,4-dinitrophenol, but was inhibited by azide, as previously reported[6]. Cyanide acted in a different manner in that with little effect on the initial rates of swelling or respiration, both reactions came to a complete stop, the extent of O_2 uptake or swelling being dependent upon the amount of cyanide in

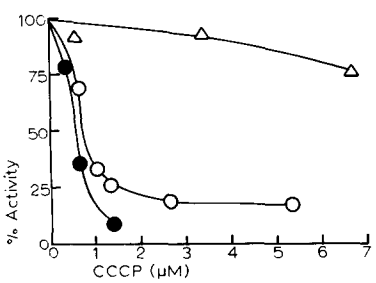

Fig. 2. The inhibition of DL-lactate-induced swelling and respiration of *A. vinelandii* by CCCP. *A. vinelandii* whole cells or cell free extract were suspended in 10 mM potassium phosphate (pH 7.4) *plus* 200 mM KCl at 24°. Swelling, % *A* (●—●) and oxygen uptake (○—○) of the whole cells, and oxygen uptake (△—△) of the cell free extract were measured, in the presence of varying concentrations of CCCP, on addition of 20 mM DL-lactate as described in METHODS.

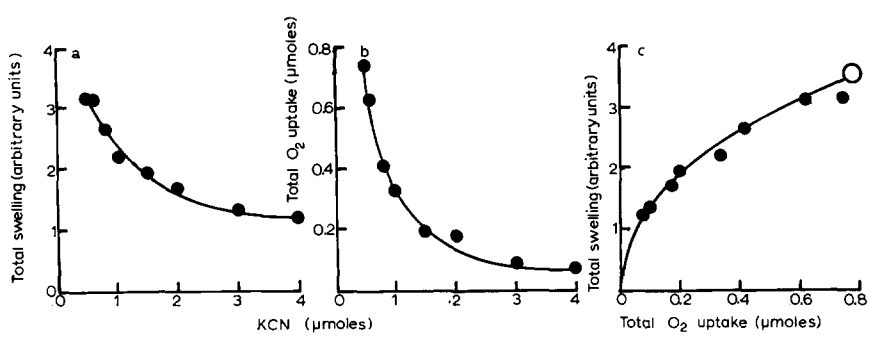

Fig. 3. The inhibition of mannitol-induced swelling and respiration by cyanide. *A. vinelandii* whole cells were suspended in 10 mM potassium phosphate (pH 7.4) *plus* 200 mM KCl. The desired concentration of cyanide was then added and the solution incubated for 10 min at 24°. 10 mM mannitol was added and the total swelling, $\%\Delta A$, (a) and oxygen uptake (b) were measured as described in METHODS. The results are also shown replotted to give the observed swelling in terms of oxygen uptake for each cyanide concentration (c). The open circle in (c) represents the swelling observed on depletion of the medium oxygen in the absence of cyanide.

the reaction mixture (Figs. 3a and 3b). On the other hand, cyanide inhibits the rate of respiration of cell-free extracts[6]. Fig. 3c is a plot of the extent of swelling with respect to the total O_2 uptake at different concentrations of cyanide in the reaction mixtures.

Proton extrusion or uptake has been observed during electron transport reactions in mitochondria[7], bacteria[8–10] or chloroplasts[11], and these can be associated with cation movements in the opposite direction[8,12] and with stimulation of proton extrusion by cations[9]. Therefore changes in medium pH and K^+ concentration were measured on addition of substrate to *A. vinelandii* lightly buffered in 4 mM Tris–HCl (pH 7.4) in the presence of selected concentrations of KCl or other salts. The change in pH observed following addition of mannitol to bacteria was greater in 10 or 200 mM KCl than with no or with 300 mM KCl. The rate of respiration was affected similarly by these KCl concentrations[2], so that the ratio of O_2 uptake to proton extrusion* remained around 5:1 (range 4.5–5.7 to 1) (Table II). The rates of respiration and proton extrusion also varied in parallel on stimulation or inhibition of the respiration by adding NaCl, $MgCl_2$, sucrose[2] or 2,4-dinitrophenol (ratio in range 4.1 to 5.5). The total decrease of pH measured was about 0.25 unit before respiration ceased due to exhaustion of O_2 in the medium.

TABLE II

MANNITOL INDUCED OXYGEN UPTAKE AND PROTON EXTRUSION AS A FUNCTION OF THE MEDIUM KCl CONCENTRATION

A. vinelandii cells were suspended in 4 mM Tris–HCl (pH 7.40) at 23° in the presence of various concentrations of KCl. Oxygen uptake and proton extrusion were measured, as described in METHODS, on addition of mannitol to a concentration of 10 mM.

KCl concn. (mM)	*Oxygen uptake (matoms/min per mg)*	*H^+ extrusion (mequiv/min per mg)*	*Ratio O/H^-*
0	249	51.6	4.8
10	368	80.1	4.6
20	472	84.7	5.6
50	480	84.7	5.7
100	456	89.7	5.1
200	476	83.8	5.7
300	224	49.6	4.5

Measurements with the K^+ electrode showed no evidence of K^+ uptake by starved cells suspended in Tris–HCl buffer (pH 7.4) containing 25–200 μM KCl. Addition of mannitol resulted in uptake of K^+ at a maximal rate of 14 nequiv/min per mg at 23°, giving a total uptake of 15–20 nequiv/mg dry weight during the interval before O_2 was exhausted. Higher total concentrations of KCl in the medium made measurements of the small changes due to uptake inaccurate. However, under the experimental conditions used the K^+ uptake was small compared with the proton extrusion.

* The lowering of the pH could as well have resulted from uptake of OH^-. For simplicity we shall refer to the change as resulting from proton extrusion.

DISCUSSION

Starved intact cells of *A. vinelandii* are impermeable to sucrose, KCl and NaCl, and addition of these solutes to a suspension of starved cells in weak buffer results in very rapid (too rapid to measure) shrinkage of the entire cell, the wall adhering closely to the membrane[2]. The addition of an oxidizable substrate to the shrunken cells results in swelling, which can be followed by measuring changes in the absorbance (light-scattering) of the suspension. This simple procedure can be used to study the transport of substances into the bacteria, and measures only osmotically active (*i.e.* not membrane-bound) substances. In *A. vinelandii* the swelling during the interval measured is accompanied by only very small changes in concentration of substances in the medium; it must result from an increase in the internal osmotic pressure of the bacteria due to an increased concentration of the substrate and its oxidation products. Rough calculations of the extent of accumulation of osmotically active substances[2] show that the increase in internal osmotic pressure is several-fold greater than the amount that could be accounted for by the substrate oxidized. There is an accumulation of substrate and its products during the interval of active respiration. This would seem to be required to keep the concentration of substrate at a level where the dehydrogenase is functional, if it is on the "inside" of the membrane, as in some other bacterial species[13].

The rate of swelling during the oxidation of substrate by *A. vinelandii* is a function of the rate of respiration. The ratio of the rate of O_2 uptake to the rate of swelling remains relatively constant through variations resulting from changes of pH, temperature and concentration of substrate, and uncouplers of oxidative phosphorylation (CCCP, 2,4-dinitrophenol, azide) are equally inhibitory to the respiration and swelling. Related observations have been made with other bacterial species. *Escherichia coli* suspended in 0.24 M KCl showed roughly similar rates of respiration and light-scattering changes on addition of some oxidizable substrates; however, the K_m for the two processes was not the same with glutamate as substrate. With *E. coli* KCl was incorporated as well as substrate[14] and the energy for the uptake of one substance could be derived from the oxidation of another substance[14,15]. The light-scattering changes in *E. coli* were claimed to result from plasmolysis and deplasmolysis, rather than from shrinking and swelling of the whole cells, as in *A. vinelandii*. MARQUIS AND GERHARDT[16] measured the uptake of a non-metabolizable amino acid analogue, α-aminoisobutyric acid by *Bacillus megaterium* (not suspended in concentrated salt or sucrose). They found the extent of non-passive uptake of α-aminoisobutyric acid to be related to the rate of respiration under various conditions; however, the respiratory rate with a given substrate was not stimulated by α-aminoisobutyric acid uptake. The uptake was inhibited by uncouplers and respiratory chain inhibitors.

Taken all together the data show that the transport of substrate into *A. vinelandii* is linked in some way to energy-yielding reactions associated with the respiratory chain. Substrate (and its oxidation products) actually accumulate, so more than one molecule of substrate can be transported per molecule of substrate oxidized. This would certainly be a necessity, since respiration is the important source of energy-yielding reactions in aerobic bacteria. The rate-limiting step in transport is some energy-yielding step associated with the respiratory chain system and in turn the transport process furnishes substrate to keep the respiration going at a constant rate

during the interval until the O_2 is exhausted. In some way the transport is controlled by the respiration and vice versa. There is no evidence that it is ATP which directly supplies the energy for transport. The rate of decrease in the ATP level in these bacteria under anaerobic conditions (when no ATP is being synthesized) is similar in the presence of β-hydroxybutyrate and lactate[3], while the rates of respiration and swelling are very different with these two substrates (Fig. 1). Although the anaerobic rate of utilization of ATP is somewhat higher in the presence of substrates than in starved cells[3], the present data would imply that this increase in utilization is not due to the utilization of ATP *per se* in transport, as previously suggested, but to other process(es) requiring the presence of substrate. Conversely, the accumulated evidence with these and other bacterial species point to a lack of the "tight coupling" of respiration to ATP synthesis (and thus the control of the respiration rate by the ratio of (ADP + P_i/ATP) which is characteristic of the systems of mammalian mitochondria[1,3,16–18].

The respiration rate of *A. vinelandii* is decreased by CCCP, 2,4-dinitrophenol and azide, while the respiration of cell-free extracts is unaffected by similar concentrations of CCCP and 2,4-dinitrophenol. CCCP uncouples respiration and phosphorylation of cell-free extracts and 2,4-dinitrophenol is also reported to have variable effects as an uncoupler in extracts[19]. Azide can act both as an uncoupler and as an inhibitor of cytochrome oxidase. These inhibitions of respiration can be explained as inhibition of transport of substrate into the cells due to lack of energy supply. The resulting deprivation of substrate at the site of the dehydrogenase inhibits respiration and consequently the energy for transport of substrate with the inhibition of the light-scattering changes. Uncoupling agents have been shown to inhibit the respiration of intact cells of *E. coli*[14,20] and *Hemophilus parainfluenzae*[21]. The effect of the uncoupling agents and 2,4-dinitrophenol on "tightly coupled" mitochondria is different; they have no effect on or stimulate respiration.

Although cyanide inhibits the respiration of cell-free extracts, its effect on the intact cells is different and also different from that of CCCP and 2,4-dinitrophenol and azide. Cyanide inhibits the total extent of swelling and O_2 uptake and this is stoichiometric with the cyanide added. Apparently cyanide specifically and firmly binds to sites or carriers of the membrane involved in transport. LUSK AND KENNEDY[22] found that 0.5 mM KCN completely inhibited the exchange of Mg^{2+} in *E. coli*.

During the swelling accompanying the oxidation of substrate, the respiration rates remained zero order until very low concentrations of oxygen were reached. This agrees with previous observations[2] that changes in the membrane during shrinkage and swelling do not affect the functioning of the membrane-bound electron transport system.

As with mitochondria[7], chloroplasts[11] or other species of bacteria[8–10], initiation of electron transport in *A. vinelandii* is accompanied by proton transport. The steady state rate of proton extrusion changes in parallel with changes in respiration rate, so that the ratio of oxygen atoms utilized to protons extruded averaged around 5:1. The net changes in the concentration of K^+ in the medium during respiration and proton extrusion was very small under the measuring conditions used. In the presence of 25–200 μM KCl, the rate of K^+ decrease was about an order of magnitude less than that of the proton extrusion. It was not possible to make accurate measurements in the presence of higher concentrations of K^+ in the medium.

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