DIRECT OBSERVATION WITH AN ELECTRODE OF UNCOUPLER-SENSITIVE ASSIMILATORY NITRATE UPTAKE BY *RHODOPSEUDOMONAS CAPSULATA*

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1. Introduction

A major deficiency in our understanding of bacterial transport is the question of how nitrate enters bacteria, to act either as an electron acceptor or as a source of nitrogen. One of the reasons for the dearth of information about nitrate transport is probably an experimental one; transport is generally studied using radioisotopes, but the radioisotope of nitrogen, ^{13}N, has a half-life of only 10 min. Despite this unhelpful property ^{13}N has been successfully used in order to study nitrate uptake into *Pseudomonas fluorescens* [1]. The bacteria were grown aerobically with nitrate as the source of nitrogen and therefore, as there was evidently repression of any transport systems associated with nitrate respiration in this denitrifying organism, the first measurements of assimilatory nitrate uptake in bacteria were made [1].

Here, we report that the assimilatory uptake of nitrate by *Rhodopseudomonas capsulata*, which does not denitrify, can be readily and continuously followed using a nitrate-specific electrode. Use of this method has shown that nitrate uptake is rapidly inhibited upon collapsing the proton electrochemical gradient across the plasma membrane either by addition of a protonophore uncoupler, or by transferring the cells from an illuminated to a darkened state. These data indicate that nitrate uptake is directly dependent on the proton electrochemical gradient rather than on the availability of intra-cellular ATP. It is also shown that nitrate uptake is rapidly but reversibly inhibited by ammonium ions, but that the ammonium analogue methylammonium causes an irreversible inhibition over the period of time that was examined.

2. Materials and methods

Rps. capsulata strain N22 was grown anaerobically under illumination with malate as carbon source as in [2] except that as nitrate was the nitrogen source $(NH_4)_2SO_4$ was omitted and replaced by sufficient KNO_3 to maintain an equivalent concentration of nitrogen in the medium. Late-exponential-phase cells were harvested and then washed and resuspended in growth medium from which nitrate had been omitted. The cells were stored on ice for up to 8 h and their bacteriochlorophyll content was measured as in [2,3].

Nitrate uptake by the cells was followed using an Orion nitrate electrode (model 93-07) in combination with a remote calomel reference electrode that made electrical contact with the reaction mixture via a sodium sulphate salt bridge. The output from the electrodes was measured with a Pye 292 meter and a chart recorder. The reaction mixtures contained 12 ml growth medium without nitrate, of which the principle components were 10 mM Na_2HPO_4, 30 mM Na-malate (pH 7.0) together with suitable quantities of cells and nitrate as noted in the legends to the figures. Illumination was from a 150 W quartz-halogen lamp the output of which was passed through 5 cm of water and one layer of Wratten 88A gelatin filter. Anaerobiosis in the electrode vessel was obtained with a stream of argon directed over the surface of the reaction mixture [4] and by passing argon through the nitrate-deficient growth medium for ⩾20 min before use. All experiments were done at 30°C.

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

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3. Results and discussion

A fresh suspension of cells did not take up nitrate in the dark, but upon illumination nitrate uptake began after a lag of ~5 min (fig.1). This uptake continued at a rate of ~330 nmol . (μmol bacteriochlorophyll)$^{-1}$. min^{-1} until the nitrate concentration fell below the range where the electrode could be conveniently calibrated (fig.1). The observation (fig.1) that there was no decrease in the rate of nitrate uptake as nitrate went to <20 μM shows that the apparent K_m for the uptake must be considerably <20 μM, and could therefore have a value close to the 7 μM estimated by a different method in *Ps. fluorescens* [1]. When the light source was removed nitrate uptake ceased within the response time of the electrode (10 s for >90% response) (fig.2), but upon re-illumination nitrate uptake was promptly restored even when the dark period had been as long as 10 min (fig.2). This uptake of nitrate is taken to be associated with assimilation of nitrogen because no uptake by cells grown with ammonium as the nitrogen source was observed.

Fig.2 also shows that nitrate uptake was completely and rapidly inhibited by low concentrations of the protonophore uncoupler FCCP. This result, together with the observation of a strict requirement for light (fig.2), is consistent with nitrate uptake being directly dependent on a proton electrochemical gradient across the plasma membrane of *Rps. capsulata*. Comparable concentrations of FCCP or darkening have been shown to collapse the membrane potential component of the gradient [4], and are expected to collapse any contribution from a pH gradient which in any case is likely to be small as the external pH of 7.0 is unlikely to be very different from intra-cellular pH [4]. An alternative explanation for the effects of darkening and of FCCP is that they reduce the availability of intracellular ATP. In principle ATP might be directly required for transport, as for example, it is in the case of glutamate transport in *Escherichia coli* [5]. A second possibility is that lack of ATP might permit the accumulation of metabolic intermediates which inhibit nitrate uptake (e.g., ammonium, see below). However, these last two explanations for the observed inhibition of nitrate uptake (fig.2) would require a

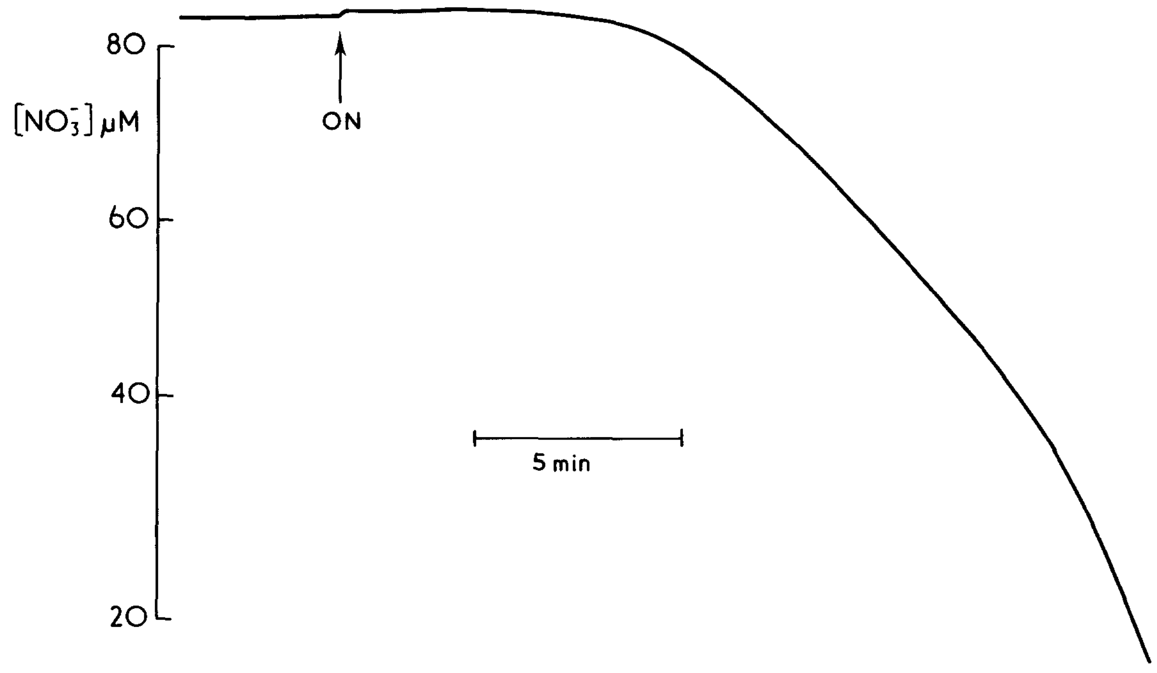

Fig.1. Light-dependent uptake of nitrate by intact cells of *Rps. capsulata*. Growth medium (12.0 ml) initially deficient in nitrate, was added to the reaction chamber (section 2). The electrode was calibrated with additions of NaNO$_3$ to give the scale shown left. Bacterial cell suspension (0.6 ml) containing 0.27 μmol bacteriochlorophyll was added and the suspension was incubated under argon for 5 min before illumination.

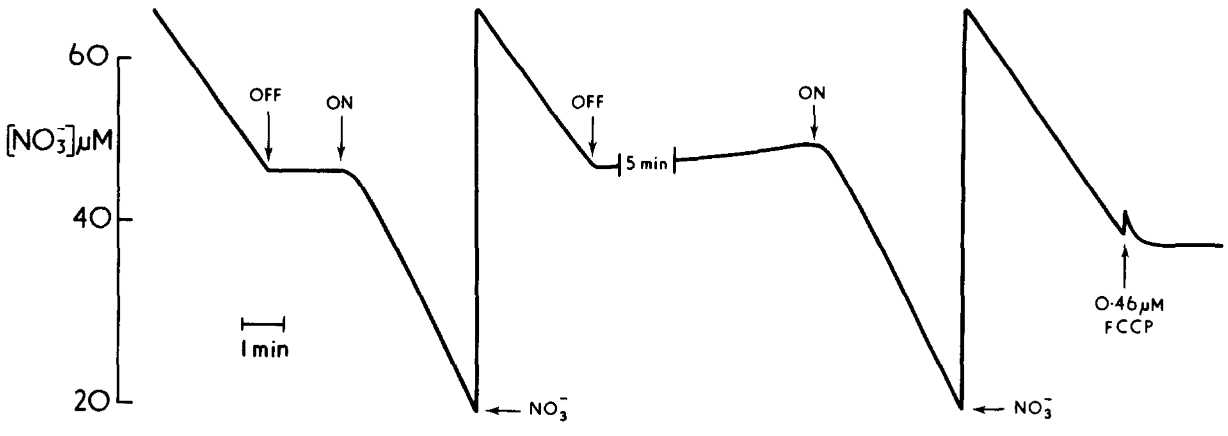

Fig.2. The energy-dependence of nitrate uptake by intact cells of *Rps. capsulata*. Conditions were as in fig.1 except that 1 ml of cells containing 0.30 μmol bacteriochlorophyll was used. The record begins after the cells had been illuminated for ~10 min. Further additions of nitrate were made as shown.

dependence on changes in ATP concentration that are not only rapid but relatively small, because the ATP content of purple bacteria decreases only very slowly upon darkening pre-illuminated cells [2,6], presumably in part because of the action of adenylate kinase.

Work with the cyanobacterium *Anacystis nidulans* [7], and with *Ps. fluorescens* [1], has shown that assimilatory nitrate uptake is inhibited by ammonium ions. Fig.3 shows that low concentrations of NH_4^+ rapidly but reversibly inhibited nitrate uptake by *Rps. capsulata*, and that addition of increasing concentra-

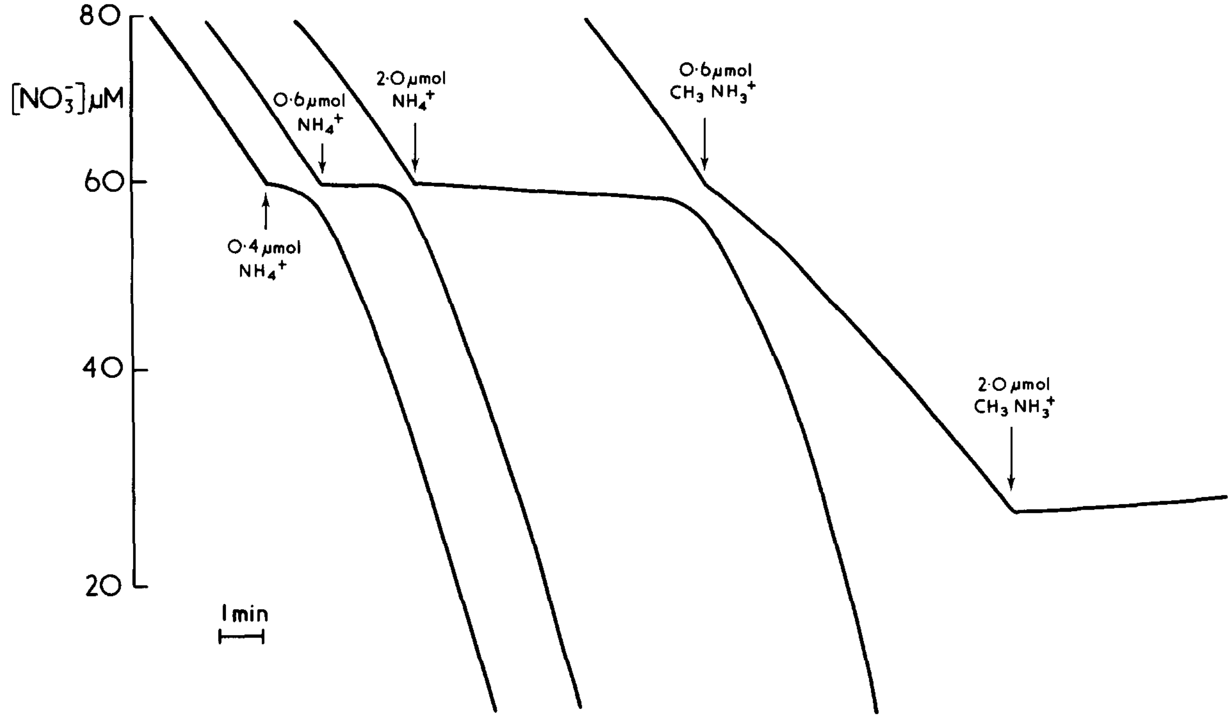

Fig.3. Inhibition of nitrate uptake by ammonium and by methylammonium. Conditions as for fig.1. All the data were obtained with the same suspension of cells. Once the nitrate concentration had fallen below 20 μM it was restored by the addition $NaNO_3$ (cf. fig.2). The NH_4^+ was added as $(NH_4)_2SO_4$, the methylammonium as the chloride salt.

tions of ammonium proportionately lengthened the duration of inhibition of nitrate uptake. An identical result was obtained when $(NH_4)_2SO_4$ was replaced by NH_4Cl, and thus the effect was clearly due to the ammonium ions and not to the nature of the added anion.

Assimilatory nitrate reductases from bacteria and blue-green algae (cyanobacteria) have been found to be insensitive to ammonium ions [1,7,8], and therefore the inhibition of nitrate uptake by NH_4^+ in *Rps. capsulata* (fig.3) is, in common with *Ps. fluorescens* [1], attributed to a direct effect on a putative nitrate-transport system. An additional observation that supports this view is that the closest structural analogue of ammonium, methylammonium, was also effective at inhibiting nitrate uptake, with 60% inhibition at 48 μM methylammonium, and essentially complete inhibition at 208 μM (fig.3). In contrast to the effect of ammonium ions, the inhibitory effect of methylammonium was not reversed over the experimental period examined (7 min), presumably because no metabolic pathway of sufficient activity is available to methylammonium in the cells grown under the conditions used here. Our interpretation of the effects of ammonium and methylammonium is that there is a high affinity binding site for ammonium ions involved in controlling nitrate uptake because only when the low concentrations of added ammonium are almost completely depleted (fig.3) is there a sharp restoration of nitrate uptake, but this binding site has a markedly lower affinity for methylammonium, and thus higher concentrations of this compound are required for inhibition.

These experiments have provided the first indications that assimilatory nitrate uptake by bacteria is directly dependent on the proton electrochemical gradient, and that this gradient is necessary for the operation of a nitrate transport system. This does not necessarily mean that nitrate is transported into cells against its concentration gradient because it is probable that rapid conversion of nitrate via nitrite to ammonium within the cells ensures that the intra-cellular concentration of nitrate remains low (cf. [1]). However, active transport of nitrate is nevertheless expected, because in illuminated cells the membrane potential is $\geqslant$160 mV (negative inside) [4] which would restrict the maximum internal nitrate concentration to $\sim 1^0/_{00}$ the external concentration if nitrate entered cells by facilitated diffusion. An added factor in this context is that the reported K_m-values for assimilatory nitrate reductase in *Rps. capsulata* are 0.18–2 mM [9], which further suggests that internal nitrate concentrations are unlikely to be determined by electrochemical equilibration of nitrate across the cell membrane. Thus the uptake of nitrate is proposed to be in symport with one or more positive charges, which need not necessarily be provided by protons, so that the energetics favour the uptake of nitrate. A final consideration is the basis for the lag observed before nitrate is taken up following initial illumination. Plausible explanations for this are that either the cells have broken down some amino acids so as to provide ammonium ions, or that the putative nitrate transport system has become inactivated during storage of the cells.

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