Arch Microbiol (1991) 155:466–472

030289339100062B

Archives of
Microbiology

Binding protein dependent transport of C4-dicarboxylates in *Rhodobacter capsulatus*

Jonathan G. Shaw and David J. Kelly

Robert Hill Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK

Received August 27, 1990/Accepted December 19, 1990

Abstract. The characteristics of malate transport into aerobically grown cells of the purple photosynthetic bacterium *Rhodobacter capsulatus* were determined. A single transport system was distinguished kinetically which displayed a K_t value of 2.9 ± 1.2 μM and V_{max} of 43 ± 6 nmol · min^{-1} · mg^{-1} protein. Competition experiments indicated that the metabolically related C4-dicarboxylates succinate and fumarate are also transported by this system. Malate uptake was sensitive to osmotic shock and evidence from the binding of radiolabelled malate and succinate to periplasmic protein fractions indicated that transport is mediated by a dicarboxylate binding protein. The activity of the transport system was studied as a function of external and internal pH and it was found that a marked activation of uptake occurred at intracellular pH values greater than 7. The use of a high affinity binding protein dependent system to transport a major carbon and energy source suggests that *Rhodobacter capsulatus* would be capable of obtaining growth sustaining quantities of C4-dicarboxylates even if these were present at very low concentrations in the environment.

Key words: Malate transport – Dicarboxylates – Periplasmic binding protein – *Rhodobacter capsulatus* – Intracellular pH

The C4-dicarboxylates malate and succinate support fast and extensive growth of many species of purple non-sulphur bacteria under both aerobic conditions in the dark and anaerobic conditions in the light. In addition to providing cell carbon, succinate can also act as a direct electron donor to the quinone pool (Zannoni and Ingledew 1983) and, under anaerobic conditions, the reduction of fumarate to succinate may act as a redox poising mechanism for the removal of excess reducing equivalents (McEwan et al. 1985).

Despite these important roles, the transport of C4-dicarboxylates has not been studied in detail in any photosynthetic bacteria. The work of Gibson (1975) did establish, however, that *Rb. sphaeroides* possessed an inducible transport system for malate, succinate and fumarate and there is evidence for a sodium driven dicarboxylate symport in *Ectothiorhodospira shaposhnikovii* (Karzanov and Ivanovsky 1980). Studies on C4-dicarboxylate transport in enterobacteria and rhizobia have shown that uptake also occurs via a common system that acts on malate, succinate and fumarate (Kay and Kornberg 1969; Finan et al. 1981). However, the molecular mechanism of transport appears to differ in the two bacterial groups. In *Rhizobium leguminosarum*, only one protein is involved in the actual transport mechanism (Ronson et al. 1984), suggesting an ion-gradient driven mechanism of energy coupling, while in *Escherichia coli* the products of at least three genes have been implicated (Lo and Sanwal 1975a). A combination of biochemical and genetic evidence strongly suggests that a periplasmic binding protein is involved in the *E. coli* system (Lo and Sanwal 1975b) but the observation that transport can be mediated efficiently by membrane vesicles alone and that the energy source for uptake may be the proton-motive force (Gutowski and Rosenberg 1975) is not in accord with this conclusion.

Periplasmic binding protein dependent transport systems consist of two hydrophobic integral membrane proteins, one or two types of membrane associated proteins and a hydrophilic protein located in the periplasm which is responsible for the initial binding of the substrate (Higgins et al. 1990). Such systems are osmotic shock sensitive and are driven by direct ATP hydrolysis (Ames and Joshi 1990). Several binding proteins have been crystallised in recent years and their structures solved (Quiocho 1990).

Offprint requests to: D. J. Kelly

Abbreviations. K_t, concentration of substrate giving half-maximal rate of transport in intact cells; Dct, protein complex encoded by *dct* genes which mediates C4-dicarboxylate transport; pmf, proton-motive force

Recently, it has been recognised that the activities of both ion-gradient driven and binding protein dependent transport systems may be subject to regulation by the intracellular pH (Poolman et al. 1987). For several substrates, an increase in internal pH above neutrality leads to a marked enhancement of the uptake rate. These effects must be allosteric in the case of binding protein dependent systems but may be catalytic or allosteric or both in the case of proton linked symport systems (Poolman et al. 1987).

In order to study the characteristics and regulation of C4-dicarboxylate transport in photosynthetic bacteria, we have used *Rhodobacter capsulatus* as a model system. Mutants deficient in transport have previously been isolated and the wild-type transport genes have been cloned by complementation (Kelly et al. 1990; Hamblin et al. 1990). In this paper, we present evidence that malate transport ocurrs via a high affinity binding protein dependent system and that the internal pH exerts a marked regulatory effect on the rate of malate uptake.

Materials and methods

Bacterial strains and growth conditions

Rhodobacter capsulatus strain 37b4 was used throughout this work and was obtained from Prof. G. Drews, University of Freiburg, FRG. For transport assays, intracellular pH determinations and the preparation of periplasmic extracts, cultures were grown aerobically at 30° C in 2.5-l shake flasks containing 250-ml volumes of minimal RCV medium (Weaver et al. 1975; Hillmer and Gest 1977) with malate as sole carbon source. For the determination of light-induced membrane potentials after osmotic shock, the cells were grown phototrophically at 30° C in 500 ml RCV medium contained in completely filled medical flat bottles at a distance of 30 cm from a 100 W tungsten lamp. Viable counts were done after plating serial dilutions on RCVYE-agar (RCV + 0.05% yeast extract + 1.5% agar).

Transport assays

After harvesting by centrifugation, late exponential phase cells were washed once in RCV medium lacking any carbon source (RCV-C) and resuspended in the same medium at a concentration of 30–40 mg cell protein ml^{-1}. The cells were stored in the dark on ice and used within 6 h. A Clarke-type oxygen electrode maintained at 30° C was filled with 2 ml RCV-C and sparged continuously with compressed air in the dark. Cells were added to a final concentration of about 0.2 mg cell protein ml^{-1} and allowed to equilibrate 5 min. Where required, various competing substrates or inhibitors were added at this stage; the ionophore nigericin was used routinely at a concentration of 1 μM at this cell density (equivalent to 10 nmol · mg cell $protein^{-1}$). The assay was started by the addition of 6 μM (22.2 KBq) [^{14}C] L-malate or [^{14}C] succinate (both uniformly labelled; specific activity 1.85–2.2 MBq μmol^{-1}). This concentration was found to give near maximal uptake rates and was used routinely. Samples (100 μl) were taken at intervals, added to 5 ml stop buffer (10 mM Na D,L-malate or succinate, 0.2 mM Na fluoracetate in 10 mM phosphate buffer, pH 7.0) and rapidly filtered onto nitrocellulose filter discs (0.45 μm pore size) which were then washed with 10 ml stop buffer, dried and scintillation counted in 3 ml Cocktail T fluid (Beckman).

Osmotic shock and isolation of periplasmic protein

Cells from a total of 1 l of culture were harvested by centrifugation at 20° C, resuspended in 40 ml of 20 mM *Tris*-HCl buffer pH 8.0 containing 0.5 M sucrose and 1.3 mM disodium EDTA (STE buffer) and shaken gently at room temperature for 15 min. After centrifugation (6,000 *g*, 15 min, 20° C), the supernatant was retained (supernatant A). The plasmolysed cell pellet was then resuspended in 20 mM *Tris*-HCl buffer pH 8.0 and again shaken gently at room temperature for 15 min. After centrifugation (15,000 *g*, 20 min, 4° C), the supernatant was removed (supernatant B) and concentrated by ultrafiltration at 4° C to a protein concentration of 3–8 mg · ml^{-1} using a Filtron stirred-cell with a 5,000 M_r cutoff. Where required, the cell pellet resulting from this step was resuspended in a minimal volume of *Tris* buffer and sonicated. After removal of unbroken cells by centrifugation (15,000 *g*, 20 min, 4° C) the supernatant (cell-free extract) was removed and stored on ice.

Dicarboxylate binding assay

Concentrated shock fluid (100 μl; 0.3–0.8 mg protein) was placed in an Eppendorf tube and incubated on ice for 10 min. [^{14}C] L-malate (7.4 KBq, final concentration 39 μM) or [^{14}C] succinate (3.7 KBq, final concentration 17 μM) was then added and incubation continued for a further 10 min. Protein was precipitated by the addition of 1 ml of ice-cold saturated ammonium sulphate solution dissolved in 10 mM acetic acid, pH 4.3, followed by immediate vacuum filtration through nitrocellulose filters (0.45 μm pore size). The filters were washed with 4 ml of cold ammonium sulphate solution, dried and scintillation counted.

Determination of internal cell volume

Cells were grown, harvested and equilibrated in RCV-C in the oxygen electrode exactly as described for transport assays, except that 4 mg of cell protein was used. In two separate experiments, either 74 KBq ^3H_2O or 14.8 KBq [^{14}C] dextran was added to the cell suspension and incubated 10 min. Duplicate 0.3-ml samples were taken, added to 0.3 ml of a silicone oil mixture (Dow Corning 200/1 grade: Dow Corning 550 grade, 1:4 v/v) and microfuged at 13,800 *g* for 2 min to pellet the cells. Samples (50 μl) were taken from the aqueous supernatant, added to 5 ml scintillation fluid and counted. The bottoms of the Eppendorf tubes containing the cell pellets were cut off, vortexed vigorously in 5 ml scintillation fluid and counted. The pellet and extracellular volumes were calculated from the ratio of ^{3}H and ^{14}C respectively in the cell pellet and supernatant. The intracellular volume was taken as the difference between the pellet and extracellular volume.

Determination of internal pH

This was done using the same batch of cells at the same protein concentration as used for the internal volume determination. As above, the experiments were performed in the oxygen electrode under conditions as similar as possible to those used for the transport assays. Cells were added to RCV-C solutions of pH values 6.0–8.0 and the same ratio of nigericin : cell protein (10 nmol · mg^{-1} protein) as used routinely for malate transport assays was also added when required. [^{14}C] U-benzoic acid (18.5 KBq) was then mixed with the cell suspension and incubated 10 min. Samples were taken, microfuged on silicone oil and counted as above. Internal pH (and hence Δ pH) was calculated using the Nernst equation and the internal cell volume.

Other methods

Malate dehydrogenase activity was assayed as described previously (Kelly 1988). Light induced membrane potentials in intact cells were

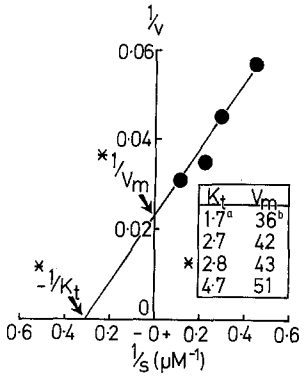

Fig. 1. Determination of K_t and V_{max} values for aerobic malate transport in intact cells of *Rhodobacter capsulatus*. Initial rates of uptake (v; nmol · min^{-1} · mg^{-1} protein) were determined at malate concentrations of 2–10 μM using four separate batches of cells. The figure shows a representative set of data which gave the kinetic parameters indicated with an *asterisk* in the inset. The data obtained with the other batches of cells is also given in the inset for comparison; K_t values [a] are in μM and V_{max}, values [b] are in nmol · min^{-1} · mg^{-1} protein

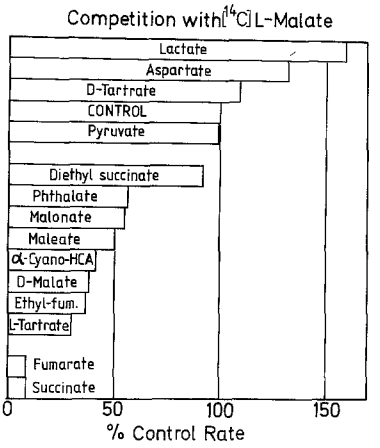

Fig. 2. Specificity of the dicarboxylate transport system as measured by substrate competition with [^{14}C] L-malate. All substrates were added to uptake assays to a final concentration of 50 μM before addition of radiolabelled malate. The initial rates of malate uptake were determined and compared to a control with no additions. The control rate was 15 nmol · min^{-1} · mg^{-1} protein

monitored under anaerobic conditions using the electrochromic response of the endogeneous carotenoids (McEwan et al. 1983) with an Aminco DW2000 dual wavelength spectrophotometer. The wavelength pair used was 528-511 nm. Bacteriochlorophyll was determined after extraction of whole cells into acetone:methanol (7:2 v/v) using an extinction coefficient of 75 mM^{-1} · cm^{-1} at 772 nm (Clayton 1963). Protein was determined by the Lowry method.

Chemicals

All chemicals were of analytical grade. Radioisotopes were obtained from Amersham International plc, England.

Results

Determination of kinetic parameters of [^{14}C] L-malate transport and substrate specificity of the dicarboxylate transport system

We have previously shown that mutants of *Rhodobacter capsulatus* which have a single Tn5 insertion in a locus designated *dct* are unable to transport malate aerobically and fail to grow on malate, succinate or fumarate under aerobic conditions in the dark (Hamblin et al. 1990). This indicates that only one dicarboxylate transport system is normally present in wild-type cells under these conditions. All the experiments described below were therefore performed with aerobically grown cells incubated under oxygen replete conditions in the dark in order to fully characterize the dct system.

The kinetic parameters of aerobic dicarboxylate transport were determined under zero-*trans* entry conditions. Figure 1 shows a representative double reciprocal plot of the dependence of the initial rate of L-malate transport on the concentration of radiolabelled substrate at an external pH of 7. K_t and V_{max} values were determined by linear regression analysis of such data using a microcomputer program. Four separate determinations using different batches of cells resulted in the range of K_t and V_{max} values shown in the inset to Fig. 1. The mean values calculated from these data were 2.9 ± 1.2 μM and 43 ± 6 nmol · min^{-1} · mg^{-1} protein respectively. In all subsequent assays, the concentration of [^{14}C] L-malate used was 6 μM ($2 \times K_t$).

The substrate specificity of the dct system was investigated by measuring the degree of competition for L-malate uptake upon inclusion of unlabelled substrate analogues in the assay mixture at a concentration of 50 μM, approximately twenty times the K_t value (Fig. 2). Of the C4-dicarboxylates, both fumarate and succinate almost completely prevented L-malate uptake but D-malate was much less effective, inhibiting the rate by about 62%. However, monoethyl fumarate was a much more potent inhibitor than diethyl succinate, emphasising the importance of the carboxyl groups in substrate recognition. Interestingly, L-tartrate (a C4-dicarboxylate with a close structural similarity to L-malate) was the most effective competitive inhibitor after fumarate and succinate but D-tartrate did not compete and actually stimulated malate uptake slightly. A stimulation of L-malate transport was also observed with aspartate and DL-lactate. Maleate, malonate and phthalate inhibited uptake by about 50% and a well characterised inhibitor of eukaryotic pyruvate transport, α-cyano-4-hydroxycinnamic acid (Halestrap and Denton 1974) also acted as an inhibitor of malate transport in this system. Importantly, however, pyruvate itself did not compete with malate uptake.

When 6 μM [^{14}C] succinate uptake was studied under identical conditions, it was found that 50 μM unlabelled malate, fumarate or succinate inhibited the rate of transport by 87%, 85% or 92% respectively.

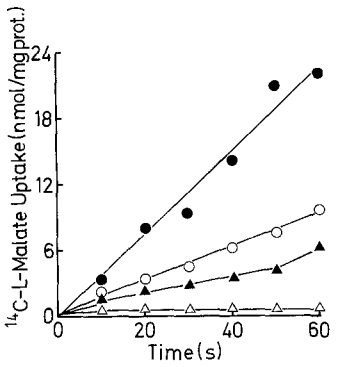

Fig. 3. Effect of osmotic shock and EDTA treatment on malate transport. Cells were subjected to osmotic shock in either sucrose-*Tris*-EDTA (STE) buffer (△——△) or sucrose-*Tris* buffer (▲——▲) and the initial rates of malate uptake were compared with unshocked cells assayed in RCV-C in the presence of 1 mM EDTA (○——○) and with unshocked control cells assayed in RCV-C with no additions (●——●)

Table 1. Distribution of malate binding activity and malate dehydrogenase (a cytoplasmic marker enzyme) in the supernatants obtained after osmotic shock of intact cells and in a cell-free extract resulting from sonication of the plasmolysed cell pellet. Supernatant A represents the fraction obtained after STE treatment of intact cells. Supernatant B represents the fraction resulting from dilution of STE plasmolysed cells in *Tris* buffer (see 'Materials and methods')

Cellular fraction	Malate dehydrogenase activity		Malate binding nmol · mg^{-1} protein)
	(nmol · min^{-1} · mg^{-1} protein)	(% of total activity)	
Experiment 1			
Cell-free extract	3417	88.3	< 0.05
Supernatant A	100	2.6	0.11
Supernatant B	353	9.1	0.81
Experiment 2			
Cell-free extract	1406	85.3	< 0.05
Supernatant A	50	3.0	0.08
Supernatant B	192	11.6	1.25

Effect of osmotic shock on transport and demonstration of dicarboxylate binding to periplasmic protein fractions

The sensitivity of malate transport to osmotic shock is illustrated in Fig. 3. In this experiment, wild-type cells were treated with a low concentration of EDTA in a hypertonic *Tris* buffered sucrose solution (STE) to induce plasmolysis and partially release the contents of the periplasm. This represents the first stage of the procedure described by Neu and Heppel (1965) for the isolation of periplasmic proteins. Such treatment (at an external pH of 7 or 8) led reproducibly to the total abolition of transport activity. Below, it is shown that the maintenance of a high internal pH is essential for transport activity. In a control experiment at an external pH of 8.0, we found that osmotic shock reduced the internal pH from 8.0 to 7.86. This decrease is insufficient (Fig. 7) to account for the effect observed. That the effect of osmotic shock was not due to cell lysis or gross metabolic disturbance was shown in several other ways. Firstly, plate counts showed that no significant loss of cell viability resulted from the STE treatment. Secondly, in separate experiments, we determined the effects of osmotic shock on the ability of photosynthetically grown cells to generate a light induced membrane potential. In one set of experiments, untreated wild-type cells generated a light induced electrochromic absorbance change at 528−511 nm of 0.009 ΔA μmol · Bchl^{-1}, while osmotically shocked cells gave a value of 0.07 ΔA μmol · Bchl^{-1}. This result indicates that shocked cells are still able to carry out photosynthesis and maintain an intact cytoplasmic membrane. It was found, however, that the addition of EDTA alone to transport assays with unshocked intact cells reduced the rate of uptake considerably (Fig. 3) but that washing the treated cells several times in fresh growth medium (minus malate) containing excess divalent cations to eliminate residual EDTA did not result in restoration of malate transport activity. Nevertheless, osmotic shock with sucrose in the *absence* of EDTA also markedly decreased the rate of malate transport (Fig. 3). Taken together, these data suggest a specific effect of osmotic shock in removing a periplasmic component involved in dicarboxylate transport.

In order to investigate further the possibility that a periplasmic binding protein may be involved in dicarboxylate transport, the degree of binding of radiolabelled malate to concentrated periplasmic protein preparations was measured using a filter binding assay (Table 1). The supernatant from STE treated cells was kept (supernatant A) and the plasmolysed cells then resuspended in a low ionic strength *Tris* buffer followed by removal of the cells and concentration of the supernatant fluid (supernatant B). This fraction contained most of the periplasmic protein, as evidenced by the characteristic brown colour due to the presence of *c*-type cytochromes. Malate dehydrogenase activity was assayed in both supernatants as a cytoplasmic marker enzyme to monitor cell lysis and was found to be low in supernatant A and only moderately increased in supernatant B. On a unit protein basis, the binding of radiolabelled malate was found to be greatest in supernatant B while much less malate bound to supernatant A protein (Table 1). That the observed binding was specific was demonstrated by the lack of binding to an equivalent concentration of bovine serum albumen and by competition experiments (Fig. 4a). Addition of an excess of unlabelled malate, succinate or fumarate to supernatant B before adding radiolabelled malate led to a decrease in counts to the same level as the BSA control but this did not occur when pyruvate was added. Similar results were obtained using radiolabelled succinate (Fig. 4b).

Regulation of dicarboxylate transport by external and internal pH

Figure 5 illustrates a series of experiments in which the dependence of the initial rate of malate transport on

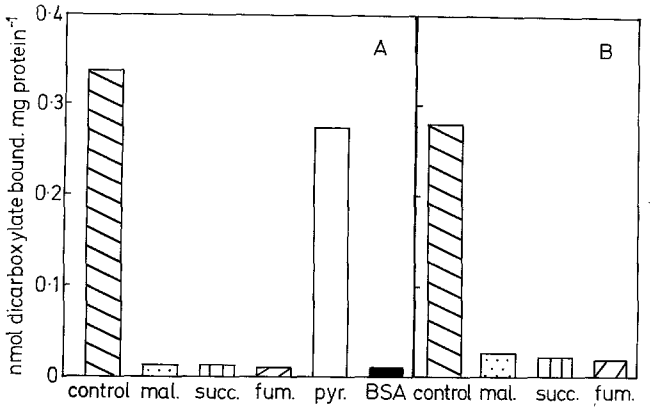

Fig. 4 A, B. Binding of C4-dicarboxylates to the periplasmic protein fraction (supernatant B) of shocked cells. In **A**, the amount of [^{14}C] L-malate bound to the periplasmic protein fraction was measured either alone (control) or in the presence of an excess (4 mM) of unlabelled malate, succinate, fumarate or pyruvate, added prior to the addition of radiolabel. The amount of malate which bound to an equivalent concentration of bovine serum albumen (BSA) was also determined. In **B**, [^{14}C] labelled succinate was used in place of malate

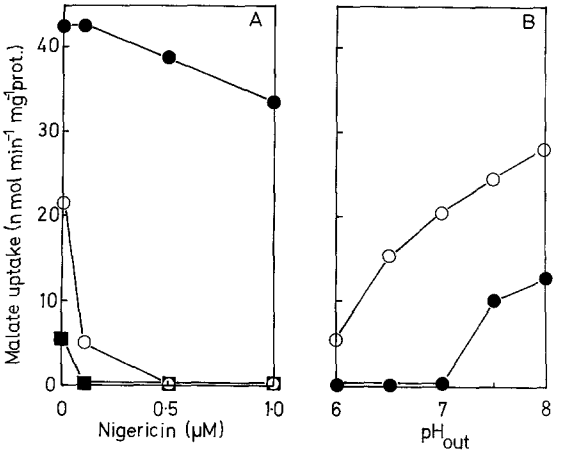

Fig. 5. A Titration of the initial rate of malate transport with the ionophore nigericin at external pH values of 6.0 (■——■), 7.0 (○——○) and 8.0 (●——●). **B** Effect of external pH on transport in the absence (○——○) and presence (●——●) of 1 μM nigericin

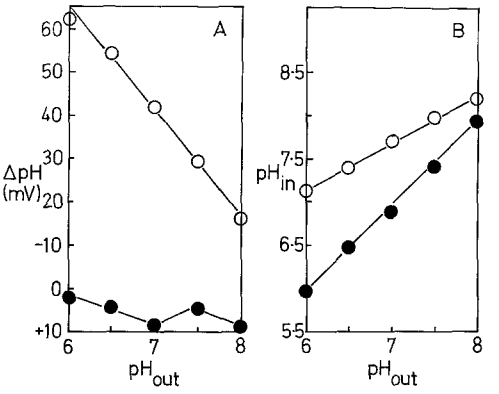

Fig. 6. A Determination of ΔpH at different external pH values in the presence (●——●) and absence (○——○) of nigericin. **B** Variation of intracellular pH as a function of external pH in the presence (●——●) and absence (○——○) of nigericin

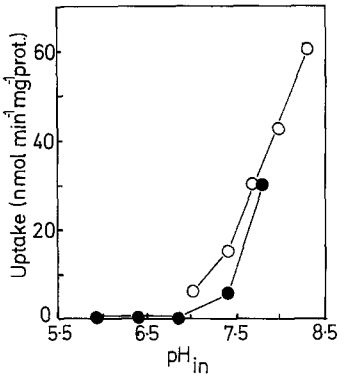

Fig. 7. Dependence of the initial rate of [^{14}C] L-malate transport on the intracellular pH value in the absence (○——○) and presence (●——●) of nigericin

external pH was determined in the presence and absence of the ionophore nigericin. The uninhibited transport rate was found to increase markedly with an increase in external pH from 6.0 to 8.0 (Fig. 5a and b). Figure 5b compares the pH dependence of transport in the presence of 1 μM nigericin with the uninhibited rate. Nigericin abolished transport activity at external pH values of less than 7.0 but not at more alkaline pH values. Titration of the rate with increasing concentrations of nigericin using a separate batch of cells (Fig. 5a) showed that at pH 6.0, 0.1 μM was sufficient to abolish transport activity and confirmed that at higher pH, malate transport was much less sensitive to this ionophore.

Nigericin catalyses an electroneutral exchange of K^+ and H^+ and this can lead to the selective dissipation of the ΔpH component of the protonmotive force with a corresponding enhancement of the membrane potential. In Fig. 6a the magnitude of ΔpH across the cytoplasmic membrane of *Rb. capsulatus* was measured by the distribution of radiolabelled benzoic acid in the presence and absence of nigericin and at different external pH values under conditions similar to those used in Fig. 5a and b. ΔpH was found to be maximal at the lowest external pH value tested (6.0) and decreased linearly with increasing external pH until it was almost zero at pH 8.0. Nigericin dissipated ΔpH at all external pH values tested. The relationship between the external and internal pH in the presence and absence of nigericin is illustrated in Fig. 6b. With a change in external pH of 2 units, the internal pH changed by about 1 unit in the absence of nigericin but the relationship became directly proportional in the presence of the ionophore.

The dependency of the rate of malate transport on the internal pH value is shown in Fig. 7. The results clearly demonstrate that above an internal pH of 7, the rate of malate uptake increases steeply but is almost zero at internal pH values less than neutrality.

Discussion

The data reported in this paper show that the uptake of malate by aerobically grown cells of *Rb. capsulatus*

proceeds by a single high-affinity transport system which also acts on succinate and fumarate. The high affinity of the Dct system is coupled with a marked specificity for dicarboxylic acid substrates. Aspartate, for example, which is structurally very similar to succinate, did not compete with malate uptake in *Rb. capsulatus* yet is a substrate for the dicarboxylate transport systems in both *Rhizobium* (Watson et al. 1988) and *E. coli* (Kay 1971). Evidence was also obtained in this study of a significant degree of stereospecificity in substrate binding, based on the results of competition experiments with D- and L-tartrate and, to a lesser extent, with D- and L-malate. These two pairs of substrates are very similar structurally and the data suggest that L-tartrate (but not the D-stereoisomer) might be transported via the dct system. However, *Rb. capsulatus*, unlike *Rb. sphaeroides*, is unable to utilise tartrate as a sole carbon source for growth (Weaver et al. 1975; Pellerin and Gest 1983). It is unlikely that a separate transport system exists for D-malate, as *dct* mutants with single Tn5 insertions were isolated on the basis of their lack of growth on a racemic mixture of D- and L-malate (Kelly et al. 1990).

Two lines of evidence strongly indicated that the Dct system in *Rb. capsulatus* is dependent on a periplasmic binding protein for activity. Firstly, malate transport was extremely sensitive to osmotic shock. This characteristic is shared by all binding protein dependent systems characterised to date but not by those driven by either an ion-gradient or exchange mechanism (Furlong 1987). We established that the effect of osmotic shock was not due to gross alteration of intracellular pH or to cell lysis and that the presence of EDTA, which does inhibit transport in unshocked cells to some degree, is not necessary to produce a significant reduction in transport activity upon plasmolysis of the cells with sucrose alone. The second line of evidence came from the demonstration of malate binding activity in the periplasmic protein fraction of shocked cells. As binding was outcompeted by succinate and fumarate but not pyruvate, we conclude that the interaction observed is specific and due to the presence of a protein which is an essential component of the dct system. However, despite the fact that STE treatment alone was sufficient to abolish malate transport, the protein released by this step did not bind a significant amount of radiolabel when compared with a cell-free extract control. Only when the bulk of the periplasmic protein was released by dilution of the plasmolysed cells in *Tris* buffer was significant malate binding activity detectable in the supernatant. These observations explain previous negative results concerning the presence of a dicarboxylate binding protein in *Rb. capsulatus* (Kelly et al. 1990). One possible explanation for this behaviour is that STE treatment disrupts the structure of the periplasm to such an extent that it destroys the normal interaction between binding protein and the intrinsic membrane protein(s) necessary for substrate translocation across the cytoplasmic membrane, but not to the point which results in large-scale protein release. The net inflow of water and expansion of the cells resulting from the subsequent resuspension in a low ionic strength buffer may then result in a release of most of the periplasmic protein (Neu and Heppel 1965; Willis et al. 1974), including the dicarboxylate binding protein.

There is now good evidence to conclude that energy coupling to binding protein dependent transport systems is achieved by the direct hydrolysis of ATP and does not require the presence of a proton-motive force (Ames and Joshi 1990). Nevertheless, perturbation of the pmf has been reported to affect binding protein dependent transport without necessarily altering the intracellular ATP concentration (Plate 1979; Ames and Joshi 1990). It has recently been recognised that this effect is due to the influence of the intracellular pH value on the activity of the transport system and not to an effect of the pmf itself (Poolman et al. 1987; Higgins et al. 1990). In this study, we used the ionophore nigericin to examine the interaction between dicarboxylate transport, the proton-motive force and internal pH. No easily interpretable relationship was apparent between transport and the ΔpH component of the pmf; nigericin dissipated ΔpH at all external pH values tested but did not abolish transport at alkaline pH values. In principle, it could be argued that this pattern reflects uptake driven solely by ΔpH at external pH values of less than neutrality (which would be nigericin sensitive) but that the driving force is the membrane potential at more alkaline external pH values where the contribution of ΔpH to the total pmf is minimal. This explanation is clearly hard to reconcile with a binding protein dependent mechanism of transport. However, when the rate of uptake is plotted against the internal pH value, either in the presence or absence of nigericin, a unique relationship is observed which appears to reflect the activation of the transport system at high internal pH. The form of this relationship is such that a relatively small increase in intracellular pH above neutrality leads to a large increase in the rate of uptake. This might have important physiological consequences during growth on dicarboxylate substrates in batch culture, as a large rise in the external pH occurs concomitantly with substrate utilisation. Regulation of transport by internal pH has been found in several other transport systems, including binding protein dependent alanine transport in a related photosynthetic bacterium, *Rb. sphaeroides* (Abee et al. 1989).

The uptake of C4-dicarboxylates has been studied most extensively in the genus *Rhizobium*, where these substrates are used as the major carbon and energy source for nitrogen fixation in the *Rhizobium*-legume symbiosis. Transport appears to be mediated by a single polypeptide, the product of the *dct*A gene, and there is no evidence for the involvement of a periplasmic binding protein. The mechanism of energy coupling has not been studied extensively but it seems likely that the proton-motive force drives uptake (Finan et al. 1981). It has also been suggested that, at least in the bacteroid, an anion exchange mechanism might operate (Maloney et al. 1990). Clearly, this is a very different mode of transport to that found in *Rb. capsulatus*, yet *Rhizobium* and *Rhodobacter* are very closely related in phylogenetic terms (Woese 1987). This distinction may be related to the fact that symbiotic rhizobia have access to a far more constant and plentiful supply of dicarboxylates than photosynthetic

bacteria, which always to accumulate such substrates from the rather low external concentrations likely to occur in natural aquatic environments. A binding protein dependent transport system would thus seem ideal in the latter case as it combines a high substrate affinity with the ability to far exceed the accumulation ratio that would otherwise be imposed by the thermodynamic limits set by the proton-motive force.

Acknowledgements. We thank the UK Science and Engineering Research Council for a grant to DJK and studentship to JGS.

References

Abee T, Wal FJ van der, Hellingwerf KJ, Konings WN (1989) Binding protein dependent alanine transport in *Rhodobacter sphaeroides* is regulated by the internal pH. J Bacteriol 171:5148–5154

Ames GF-L, Joshi AK (1990) Energy coupling in bacterial periplasmic permeases. J Bacteriol 172:4133–4137

Clayton RK (1963) Toward the isolation of a photochemical reaction centre in *Rhodopseudomonas sphaeroides*. Biochim Biophys Acta 75:312–323

Finan TM, Wood JM, Jordan DC (1981) Succinate transport in *Rhizobium leguminosarum*. J Bacteriol 148:192–202

Furlong CE (1987) Osmotic shock sensitive transport systems. In: Neidhardt F et al. (eds) *Escherichia coli* and *Salmonella typhimurium*. ASM Press, Washington DC, pp 768–796

Gibson J (1975) Uptake of C4-dicarbxylates and pyruvate by *Rhodopseudomonas sphaeroides*. J Bacteriol 123:471–480

Gutowski SJ, Rosenberg H (1975) Succinate uptake and related proton movements in *Escherichia coli* K12. Biochem J 152:647–654

Halestrap AP, Denton RM (1974) Specific inhibition of pyruvate transport in rat liver mitochondria and human erythrocytes by α-cyano-4-hydroxycinnamate. Biochem J 138:313–316

Hamblin MJ, Shaw JG, Curson JP, Kelly DJ (1990) Mutagenesis, cloning and complementation analysis of C4-dicarboxylate transport genes from *Rhodobacter capsulatus*. Mol Microbiol 4:1567–1574

Higgins CF, Gallagher MP, Hyde SC, Mimmack ML, Pearce SR (1990) Periplasmic binding protein-dependent transport systems: the membrane associated components. Philos Trans R Soc Lond [Biol] 326:353–365

Hillmer P, Gest H (1977) H_2 metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: H_2 production by growing cultures. J Bacteriol 129:724–731

Kay WW (1971) Two aspartate transport systems in *Escherichia coli*. J Biol Chem 246:7373–7382

Kay WW, Kornberg HL (1969) Genetic control of the uptake of C4-dicarboxylic acids by *Escherichia coli*. FEBS Lett 3:93–96

Karzanov VV, Ivanovsky RN (1980) Sodium dependent succinate uptake in purple bacterium *Ectothiorhodospira shaposhnikovii*. Biochim Biophys Acta 598:91–99

Kelly DJ (1988) Gram-positive type citrate synthase in the thermophilic green gliding bacterium *Chloroflexus aurantiacus*. FEMS Microbiol Lett 49:39–42

Kelly DJ, Hamblin MJ, Shaw JG (1990) Physiology and genetics of C4-dicarboxylate transport in *Rhodobacter capsulatus*. In: Drews G, Dawes E (eds) Molecular biology of membrane-bound complexes in phototrophic bacteria. Plenum Press, New York, pp 453–462

Lo TCY, Sanwal BD (1975a) Genetic analysis of mutants of *Escherichia coli* defective in dicarboxylate transport. Mol Gen Genet 140:303–307

Lo TCY, Sanwal BD (1975b) Isolation of the soluble substrate recognition component of the dicarboxylate transport system of *Escherichia coli*. J Biol Chem 250:1600–1602

Maloney PC, Ambudkar SV, Anantharam V, Sonna LA, Varadhachary A (1990) Anion exchange mechanisms in bacteria. Microbiol Rev 54:1–17

McEwan AG, Ferguson SJ, Jackson JB (1983) Electron flow to dimethylsulphoxide or trimethylamine-*N*-oxide generates a membrane potential in *Rhodopseudomonas capsulata*. Arch Microbiol 136:300–305

McEwan AG, Cotton NPJ, Ferguson SJ, Jackson JB (1985) The role of auxiliary oxidants in the maintenance of a balanced redox poise for photosynthesis in bacteria. Biochim Biophys Acta 810:140–147

Neu HC, Heppel LA (1965) The release of enzymes from *Escherichia coli* by osmotic shock and duration the formation of spheroplasts. J Biol Chem 240:3685–3692

Pellerin NC, Gest H (1983) Diagnostic features of the photosynthetic bacterium *Rhodopseudomonas sphaeroides*. Curr Microbiol 9:339–344

Plate CA (1979) A requirement for membrane potential in active transport of glutamine by *Escherichia coli*. J Bacteriol 137:221–225

Poolman B, Driessen AJM, Konings WN (1987) Regulation of solute transport in streptococci by external and internal pH values. Microbiol Rev 51:498–508

Quiocho FA (1990) Atomic structures of periplasmic binding proteins and the high-affinity active transport systems in bacteria. Philos Trans R Soc [Biol] 326:341–351

Ronson CW, Astwood PM, Downie JA (1984) Molecular cloning and genetic organisation of C4-dicarboxylate transport genes from *Rhizobium leguminosarum*. J Bacteriol 160:903–909

Watson RJ, Chan Y-K, Wheatcroft R, Yang A-F, Han S (1988) *Rhizobium meliloti* genes required for C4-dicarboxylate transport and symbiotic nitrogen fixation are located on a megaplasmid. J Bacteriol 170:927–934

Weaver PF, Wall JD, Gest H (1975) Characterization of *Rhodopseudomonas capsulata*. Arch Microbiol 105:207–216

Willis RC, Morris RG, Ciragoklu C, Schellenberg GD, Gerber NH, Furlong CE (1974) Preparation of the periplasmic binding proteins from *Salmonella typhimurium* and *Escherichia coli*. Arch Biochem Biophys 161:64–75

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271

Zannoni D, Ingledew WJ (1983) A functional characterisation of the membrane bound iron sulphur centres of *Rhodopseudomonas capsulata*. Arch Microbiol 135:176–181