Transport of glycine betaine in the extremely haloalkaliphilic sulphur bacterium Ectothiorhodospira halochloris

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The effect of osmotic stress on the transport of the compatible solute glycine betaine was examined in Ectothiorhodospira halochloris, an extremely haloalkaliphilic, phototrophic sulphur bacterium. Kinetic data indicated that E. halochloris possesses an active transport system for glycine betaine which is saturable and exhibits Michaelis–Menten kinetics. Experiments with chloramphenicol-treated cells (50 μg per ml of cell suspension) indicated that the transport system is constitutive and might be activated by a change in osmotic pressure. The uncouplers carbonyl cyanide m-chlorophenylhydrazone (CCCP) and carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (CCFP) totally blocked uptake at concentrations of 25 μM and 100 μM, respectively. The system was insensitive to the cytochrome oxidase inhibitor sodium azide (1 mM), the respiratory chain inhibitor potassium cyanide (1 mM) and the glycolysis inhibitor iodoacetate (1 mM). The ionophore nigericin (50 μM) had the greatest inhibitory effect, completely abolishing uptake, while monensin (100 μM) caused 80% inhibition. Glycine betaine transport possessed considerable structural specificity: proline betaine was the most effective competitor and trigonelline and dimethylglycine exerted inhibition to a lesser extent. Transport in the dark was at a greatly reduced rate. These results collectively implied that the specific transport of glycine betaine might be driven by the electrochemical proton gradient generated by anaerobic photosynthesis.

Introduction

Micro-organisms have evolved different strategies in responding to decreased water availability in their natural habitats. Osmotic equilibrium is maintained by either electrolytes (salts) requiring salt-adapted cellular structures or the accumulation of compatible solutes (Brown, 1976). Compatible solutes may be taken up from the surrounding medium or they may be synthesized by the bacteria themselves.

The quaternary amine glycine betaine (N,N,N-trimethylglycine) is an important compatible solute not only in extreme halophiles (Galinski & Trüper, 1982) but also in Gram-negative, non-halophilic eubacteria (Cairney et al., 1985a, b), moderate halophiles (Imhoff & Rodriguez-Valera, 1984) and halotolerant microorganisms (Ken-Dror et al., 1986). Currently, only three groups seem to be capable of de novo betaine synthesis: cyanobacteria (Borowitzka, 1980; Mohammad et al., 1983; Gabbay-Azaria et al., 1988), some anaerobic photosynthetic bacteria (genus Ectothiorhodospira) and Actinopolyspora halophila (Severin et al., 1992). Transport systems for glycine betaine have been described in Escherichia coli, Salmonella typhimurium and Klebsiella pneumoniae (Le Rudulier & Bouillard, 1983; Le Rudulier et al., 1982), in Rhizobium meliloti (Fougère & Le Rudulier, 1990), Rhodobacter sphaeroides (Abee et al., 1990), Lactobacillus acidophilus (Hutkins et al., 1987) and Azospirillum brasilense sp7 (Riou et al., 1991), as well as in some methanogenic bacteria (Robertson et al., 1990).

In E. coli and S. typhimurium, two genetically distinct transport systems were identified: a high-affinity system encoded by proU and a low-affinity system encoded by proP (Cairney et al., 1985a; Barron et al., 1987). In R. meliloti (Le Rudulier et al., 1991), A. brasilense sp7 (Riou et al., 1991) and E. coli (May et al., 1986) a periplasmic

Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; CCFP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; CDNB, 1-chloro-2,4-dinitrobenzene; DCCD, N,N'-dicyclohexylcarbodiimide; DNP, 2,4-dinitrophenol; PCMB, p-chloromercuribenzoate.
binding protein is involved in the transport of glycine betaine.

*Ectothiorhodospira halochloris*, an extremely haloalkali-phile phototrophic sulphur bacterium, uses glycine betaine as its main compatible solute in addition to betaine (Galiniski & Trüper, 1982). Glycine betaine cannot be used as carbon, nitrogen or energy source, and it cannot be stored as reserve material. Only binding protein is involved in the transport of glycine betaine changes. The current study characterizes the glycine betaine transport system in *E. halochloris*. To provide evidence for the existence of this system we examined saturation kinetics, substrate specificity and the influence of metabolic inhibitors.

**Methods**

Organism and culture conditions. *Ectothiorhodospira halochloris* strain DSM 1059 was grown anaerobically at 40 °C in 1 litre screw-cap bottles at a light intensity of 10000 lux in a medium according to Ihmoff & Trüper (1977). Acetate and bicarbonate were used as carbon sources. For transport experiments, medium containing NaCl, NaHCO₃, Na₂CO₃, Na₂SO₄ and Na₂S only at the appropriate concentrations was used. For determination of the pH optimum, the ratio of Na₂CO₃ to NaHCO₃ was varied and, if necessary, the pH adjusted with H₂SO₄ or NaOH.

Preparation of [¹⁴C]glycine betaine. [1,2-¹⁴C]Choline was prepared from [1,2-¹⁴C]choline as described by Ikuta et al. (1977). [¹⁴C]Choline was enzymically oxidized by the enzyme choline oxidase (EC 1.1.3.17) from an *Achromobactena* sp. to form [¹⁴C]glycine betaine, the reaction mixture was ultrafiltrated (Millipore Ultrafree MC, 10000 M.W. filter unit). The filtrate was incubated twice with 150 mg of ion-exchange resin (Galinski, 1987) to separate un-oxidized [¹⁴C]choline from [¹⁴C]glycine betaine. The amount of [¹⁴C]glycine betaine was determined in scintillation vials containing 4 ml of scintillation fluid (Lumagel LSC Cocktail, Baker) in a Beckman liquid scintillation counter (Beckman LS 230 liquid scintillation system).

This result was confirmed by HPLC using a Nucleosil 5 NH₂ (Macherey and Nagel, Düren, Germany) column and acetonitrile/water (70:30, v/v) as solvent. Non-radioactive glycine betaine was monitored using a LDC/Milton Roy HPLC unit with refractive index monitor (model 1109) and integrator (CI-10).

Transport assays. When the culture density reached an OD₆₅₀ of 1.2 to 1.3 (late exponential growth phase), 25 ml (0.5 mg protein ml⁻¹) of cell suspension was equilibrated for 30 min in a glass vial (N₂, 40 °C, 10000 lux). Centrifugation was avoided because the cells are sensitive to oxygen. The betaine content of the medium was determined by HPLC (Galinski, 1987) and the ammonium reineckate method (Cromwell & Rennie, 1953) to exclude any interference from glycine betaine already present in the cell suspension. If not stated otherwise, the cells were shocked from 2.1 M-NaCl to 3.1 M-NaCl either by use of transport medium (25 ml, prewarmed and gassed) of the appropriate salt concentration or as confirmation of the results by addition of solid NaCl. Temperature and pH in the transport experiments were identical with the growth conditions (40 °C, pH 8.7) to avoid changing too many parameters. Uptake experiments were started by the addition of shock medium and substrate; if not stated otherwise, 1 µM-[¹⁴C]glycine betaine and a defined amount of non-radioactive glycine betaine were used as substrate.

The reaction was terminated by rapid filtration through filters (Whatman GF/F) and the cells were then quickly washed twice with 2 ml of medium of a corresponding salt concentration. The filters were solubilized in scintillation vials containing 4 ml of scintillation fluid.

All data presented are mean values derived from duplicates of three experiments. Unless indicated otherwise, results agreed within less than 5%. The results are expressed as nmol glycine betaine (mg protein)⁻¹·h⁻¹; protein was determined by the bichinchonic acid method (BCA; Pierce, 1989).

In inhibition assays, the cells were pre-incubated with inhibitor for 30 min before addition of substrate (1 µM-[¹⁴C]glycine betaine, 4 mM-non-radioactive glycine betaine). Addition of chloramphenicol (50 µg ml⁻¹) required 1 h pre-incubation, PCMB was incubated for 10 min and CDNB for 15 min. If ethanol (no more than 2% of the cell suspension) was used as solvent (for ionophores and DCCD) the effect of ethanol itself was also tested. Detailed information about the effect of ionophores and DCCD on *E. halochloris* is provided by Meyer (1987).

The influence of dark conditions was tested by wrapping the vial with aluminium foil during pre-incubation and uptake measurements.

In competition experiments, a mixture of labelled substrate (1 µM-[¹⁴C]glycine betaine) and unlabelled analogue was added.

In order to test the range of salt stress that *E. halochloris* is able to stand without cell leakage, salt-shocked cell suspensions were centrifuged after different times and the protein content of the supernatant was measured by the method of Warburg & Christian (1941) and by the BCA method.

Chemicals. [1,2-¹⁴C]choline [72 mCi mmol⁻¹ (266 MBq mmol⁻¹)] was obtained from DuPont. Choline oxidase, glycine betaine, glycine betaine aldehyde, choline, trigonelline, γ-butyrobetaine, γ-amino-n-butyrate, l-alanine, 2,4-dinitrophenol, iodoacetate, chloramphenicol, sodium azide, nigericin, valinomycin, monensin, CCCP, CCFP, PCMB, CDNB, 1-chloromercuriphenyl sulphonic acid were purchased from Sigma. Potassium cyanide was purchased from E. Merck.

Proline betaine (stachydrine) was kindly provided by D. Le Rudulier, University of Nice, France.

**Results and Discussion**

Growth in the presence of exogenous glycine betaine and effect of increased osmolarity

Addition of 5 mM-glycine betaine to the growth medium stimulated the growth rate of *E. halochloris*, especially at high salt concentrations. For example, at 2.4 mM-NaCl there was a 9% stimulation (0.054 h⁻¹ to 0.059 h⁻¹) and at 3.8 mM-NaCl a 19% stimulation (0.031 h⁻¹ to 0.037 h⁻¹). Choline and glycine, considered as possible biosynthetic precursors of glycine betaine (Oren, 1990), had no effect. *E. halochloris* was able to stand sudden salt changes...
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Fig. 1. Time-dependent uptake of glycine betaine by E. halochloris, performed as described in Methods with 4 mM-glycine betaine at 40°C and pH 8-7. The insert shows transport during the first 2 min.

Fig. 2. Kinetics of glycine betaine transport by E. halochloris. Uptake was measured as described in Methods at 40°C and pH 8-7.

(upshock) of 1.0 M-NaCl without obvious cell damage (release of proteins). For calculating the cytoplasmic solute concentrations, cytoplasmic volume values of 257 μl (g pellet wt)^-1 (2.1 M-NaCl) and 210 μl (g pellet wt)^-1 (3.1 M-NaCl) were used (Galinski, 1986). HPLC measurements yielded a value of 150 g glycine betaine (kg cytoplasmic water)^-1 (equivalent to 1.0 molal) for cells grown at 2.1 M-NaCl and of 196 g glycine betaine (kg cytoplasmic water)^-1 (equivalent to 1.6 molal) at 3.1 M-NaCl.

Active glycine betaine transport and its kinetics

Cells of E. halochloris grown anaerobically in the light were able to take up glycine betaine against a concentration gradient after osmotic upshock (2.1 M- to 3.1 M-NaCl). Glycine betaine was not metabolized further. No measurable transport of glycine betaine was observed in the absence of osmotic stress.

Time-dependent measurement of uptake was linear for only 2 min (Fig. 1). The initial uptake rate at 40°C, pH 8-7, and 4 mM-glycine betaine was 29 ± 1 nmol min^-1 (mg protein)^-1, reaching a level of 288 ± 8 nmol (mg protein)^-1 after 20 min. Transport saturated with respect to substrate concentration (Fig. 2) and followed Michaelis-Menten kinetics. The \( K_m \) value (Hanes, 1932) was 60 ± 2 μM and the maximal velocity \( (V_{max}) \) was 29 ± 1 nmol min^-1 (mg protein)^-1. Control experiments with solid NaCl confirmed these results. For all studies, a saturating concentration of 4 mM-glycine betaine was used. The transport system had a pH optimum of pH 9.1, with more than 80% activity between pH 8.1 and pH 9.4, and an optimum temperature of 40°C, with more than 80% activity between 32°C and 52°C. The theoretically calculated amount of 270 ± 10 nmol glycine betaine (mg protein)^-1 necessary to balance the upshock from 2.1 M- to 3.1 M-NaCl was shown to agree with the value of 288 ± 8 nmol glycine betaine (mg protein)^-1 determined during uptake experiments. The physiological examinations and the kinetic data indicate the involvement of a transport protein which is activated by osmotic changes.

Transport against a gradient requires an expenditure of energy. To investigate the source of energy, various metabolic inhibitors were tested (Table 1).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc</th>
<th>Percentage inhibition</th>
</tr>
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<tbody>
<tr>
<td>Chloramphenicol</td>
<td>50 μg ml^-1</td>
<td>0</td>
</tr>
<tr>
<td>CCCP</td>
<td>25 μM</td>
<td>100</td>
</tr>
<tr>
<td>CCFP</td>
<td>25 μM</td>
<td>82</td>
</tr>
<tr>
<td>DCCD</td>
<td>100 μM</td>
<td>100</td>
</tr>
<tr>
<td>2,4-DNP</td>
<td>2 mM</td>
<td>38</td>
</tr>
<tr>
<td>KCN</td>
<td>1 mM</td>
<td>5</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>1 mM</td>
<td>9</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>1 mM</td>
<td>0</td>
</tr>
<tr>
<td>CDNB</td>
<td>0.5 mM</td>
<td>94</td>
</tr>
<tr>
<td>PCMB</td>
<td>0.5 mM</td>
<td>31</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>45 μM</td>
<td>26</td>
</tr>
<tr>
<td>100 μM</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Nigericin</td>
<td>50 μM</td>
<td>100</td>
</tr>
<tr>
<td>Memensin</td>
<td>15 μM</td>
<td>48</td>
</tr>
<tr>
<td>50 μM</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>100 μM</td>
<td>80</td>
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</tbody>
</table>

Table 1. Effect of potential metabolic inhibitors on glycine betaine transport by E. halochloris

If not stated otherwise, cells were pre-incubated for 30 min with the inhibitor before initiation of uptake (with 4 mM-glycine betaine, 40°C, pH 8-7) as described in Methods. For control cells (not exposed to inhibitor) 100% activity was 29 nmol min^-1 (mg protein)^-1.
Addition of the uncouplers CCCP (25 μM) and CCFP (100 μM), which separate photosynthetic electron transport from photophosphorylation, totally abolished glycine betaine transport. Addition of CCFP (25 μM) after 1 min of accumulation stopped further uptake, but did not cause efflux for the next 20 min. The glycolysis inhibitor iodoacetate (1 mM) had no effect; neither did the cytochrome oxidase inhibitor sodium azide (1 mM) nor the respiration inhibitor potassium cyanide (1 mM). Ethanol, which was used as solvent for some inhibitors, had no effect on transport.

The sulphhydryl reagents CDNB (0-5 mM) and PCMB (0-5 mM) were added to cells which had accumulated glycine betaine for 4 min. While PCMB had no effect for the next 20 min, further uptake was inhibited by CDNB, and there was an efflux of glycine betaine for the next 50 min (Fig. 3). Pre-incubation with CDNB caused 94% inhibition, whereas PCMB caused only 21% inhibition. The results obtained with the sulphhydryl reagents PCMB and CDNB could be interpreted on the basis of the results obtained by Koo et al. (1991). The rapid loss of glycine betaine in S. typhimurium after addition of PCMB led to the postulation of a system that cannot take up glycine betaine and proline, but which, when modified by PCMB, can bring about rapid efflux of these solutes. In E. halochloris an efflux of accumulated glycine betaine was observed after addition of CDNB. The ability of E. halochloris to excrete glycine betaine supports the results obtained by Tschichholz & Trüper (1990). Further experiments are necessary to prove the existence of either one system for uptake and release or of two distinct systems.

Nigericin (50 μM), an electroneutral K+/H+ antibiotic ionophore, totally abolished glycine betaine transport. Monensin (50 μM), an electroneutral Na+/K+ exchanger, also inhibited transport, but to a lesser extent (54%).

Valinomycin (45 μM), a K+-specific ionophore, led to a 26% inhibition of glycine betaine uptake. After 30 min pre-incubation of photosynthetically active E. halochloris in the dark, glycine betaine transport (40 °C, pH 8-7, 4 mM-substrate) was lowered to 36% [100% activity was 29 ± 1 nmol min⁻¹ (mg protein)⁻¹], reaching a plateau of 74% inhibition after 1 h. Supplementing the transport assay with 1 mM-ATP had no influence on the transport rate. ATP might not be used directly as an energy source but further studies using cytoplasmic membrane vesicles will be necessary to determine the permeability of the cells to ATP. These results imply that the uptake of glycine betaine is an energy-dependent process. The electrochemical proton gradient, generated by anaerobic photosynthesis, is the main driving force for glycine betaine transport. However, energetic interpretations are made difficult by the fact that any one of the inhibitors used might inhibit various metabolic processes. E. halochloris seems to be able to react to osmotic changes either by active transport within minutes or by new biosynthesis within hours. Active transport may be an efficient adaptive mechanism which allows E. halochloris to tolerate drastic changes in water potential due to evaporation and dilution by rain and flooding in their natural habitats, such as alkaline soda lakes (Imhoff et al., 1978), salt lakes and marine sources (Grant et al., 1979; Matheron & Baulaigue, 1972). This strategy was also suggested for some cyanobacteria that synthesize glycine betaine (Moore et al., 1987).

Table 2. Effect of various compounds on [14C]glycine betaine uptake by E. halochloris

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percentage inhibition at:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>40 μM</td>
</tr>
<tr>
<td>Glycine betaine</td>
<td>35.5±3.7</td>
</tr>
<tr>
<td>Proline betaine</td>
<td>228±1.1</td>
</tr>
<tr>
<td>Dimethylglycine</td>
<td>198±0.6</td>
</tr>
<tr>
<td>Trigonelline</td>
<td>203±1.7</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Glycine betaine aldehyde</td>
<td>2.4±0.1</td>
</tr>
<tr>
<td>y-Butyrobetaine</td>
<td>2.7±0.9</td>
</tr>
<tr>
<td>γ-Amino-n-butyrate</td>
<td>-3.5±0.9</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>-25±0.5</td>
</tr>
<tr>
<td>Choline</td>
<td>-59±0.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>-74±0.5</td>
</tr>
<tr>
<td>Proline</td>
<td>-49±1.0</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of PCMB and CDNB on accumulation of glycine betaine by E. halochloris. The sulphhydryl reagents (0-5 mM) were added (arrow) after 4 min accumulation of glycine betaine. Accumulation was measured as described in Methods at 40 °C, pH 8-7, with 2 mM-glycine betaine. ○, Control; ◐, 0.5 mM-PCMB; Δ, 0.5 mM-CDNB.
Betaine transport by *Ectothiorhodospira halochloris*

Glycine betaine was measured in the presence of radioactive competitors (Table 2). Uptake of radioactive betaine rose linearly with the glycine betaine concentration (1 mM) and 40- and 2000-fold in excess of the glycine betaine concentration (1 μM).

Transport was not affected by L-alanine, glycine betaine aldehyde and γ-amino-n-butyrate. Choline, glycine and sarcosine caused slight stimulation of transport, but the most notable stimulation was found with proline (2000-fold excess, 24% stimulation). The most competent negative effector was proline betaine, with 66% inhibition at a concentration of 2 mM, whereas dimethylglycine and trigonelline caused only 29% and 32% inhibition at the same concentration. With respect to the structural specificity of the uptake system (Fig. 4), a carboxyl group seems to be of great importance because glycine betaine aldehyde had no effect whereas choline stimulated uptake (9% at 2 mM). Glycine (without any methyl group) stimulated glycine betaine uptake, whereas dimethylglycine (2 mM) caused 29% inhibition. Proline had no inhibitory effect whereas proline betaine, which possesses two methyl groups, caused the greatest inhibition. γ-Butyrobetaine, choline and L-alanine, possessing more backbone carbon atoms than glycine betaine, had no inhibitory effects.

These results indicate that glycine betaine uptake shows considerable structural specificity, especially with respect to the presence of a carboxyl group and the constellation of the carboxyl to the methyl group. Chemical modification of the relevant carboxyl group might reveal more about its importance for glycine betaine recognition. Rolland & Tristram (1975) reported a proline transport system in *E. coli*, in which a carboxyl group binds by a hydrogen bond to the protein binding site.

**Constitutivity of the glycine betaine transport system**

The immediate stimulation of transport after osmotic upshock indicated that the system is activated and not induced by osmotic stress. Addition of chloramphenicol (50 μg ml⁻¹), which inhibits de novo protein synthesis and cell growth, had no effect on uptake, indicating that the transport system is constitutive.

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**References**


