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

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(Received 13 March 1992; revised 18 June 1992; accepted 23 June 1992)

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 quarternary 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.*, 1985*a, 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.*, 1985*a*; 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

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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 haloalkaliphilic phototrophic sulphur bacterium, uses glycine betaine as its main compatible solute in addition to ectoine and trehalose (Galinski & Trüper, 1982). Glycine betaine cannot be used as carbon, nitrogen or energy source, and it cannot be stored as reserve material. Only its extrusion in response to osmotic stress (downshock) has been investigated (Tschichholz & Trüper, 1990). The contribution of glycine betaine biosynthesis during adaptation to osmotic changes in comparison to non-stressed cells was investigated by Galinski (1986). Biosynthesis that supplements glycine betaine content after osmotic upshock became obvious after 3 h. The question raised was whether any fast responses like transport processes exist to balance sudden osmotic 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 Imhoff & 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]Glycine betaine 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 *Alcaligenes* sp. To purify [¹⁴C]glycine betaine, the reaction mixture was ultrafiltrated (Millipore Ultrafree MC, 10000 NMLW 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; protein was determined by the bicinchoninic 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 [7.2 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 M-NaCl there was a 9% stimulation (0.054 h⁻¹ to 0.059 h⁻¹) and at 3.8 M-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

Fig. 1

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

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-

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) $^{-1}$.

Inhibitor	Concn	Percentage inhibition
Chloramphenicol	50 $\mu\text{g ml}^{-1}$	0
CCCP	25 μM	100
CCFP	25 μM	82
	100 μM	100
DCCD	100 μM	30
2,4-DNP	2 mM	38
KCN	1 mM	5
Sodium azide	1 mM	9
Iodoacetate	1 mM	0
CDNB	0.5 mM	94
PCMB	0.5 mM	31
Valinomycin	45 μM	26
	100 μM	48
Nigericin	50 μM	100
Monensin	14.5 μM	48
	50 μM	54
	100 μM	80

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. The high intracellular glycine betaine content, as determined by HPLC, implied that uptake must have occurred against an intracellular concentration gradient. Transport against a gradient requires an expenditure of energy. To investigate the source of energy, various metabolic inhibitors were tested (Table 1).

Fig. 3. Effect of PCMB and CDNB on accumulation of glycine betaine by *E. halochloris*. The sulphydryl 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; △, 0.5 mM-CDNB.

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 sulphydryl 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 sulphydryl 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%).

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

Uptake experiments were performed as described in Methods with 1 µM-[¹⁴C]glycine betaine at 40 °C and pH 8.7 with the 'competitor compound' present at 40 µM or 2 mM. Results are expressed as a percentage of the non-inhibited uptake rate for 1 µM-glycine betaine: 100% was 0.078 nmol min⁻¹ (mg protein)⁻¹.

Compound	Percentage inhibition at:	
	40 µM	2 mM
Glycine betaine	35.5 ± 3.7	91.2 ± 4.7
Proline betaine	22.8 ± 1.1	65.7 ± 5.7
Dimethylglycine	19.8 ± 0.6	28.9 ± 2.2
Trigonelline	20.3 ± 1.7	31.5 ± 2.6
L-Alanine	0.8 ± 0.1	2.5 ± 0.2
Glycine betaine aldehyde	2.4 ± 0.1	-0.3 ± 0.1
γ-Butyrobetaine	2.7 ± 0.9	5.1 ± 0.2
γ-Amino-n-butyrate	-3.5 ± 0.9	0.6 ± 0.1
Sarcosine	-2.5 ± 0.5	-7.2 ± 2.2
Choline	-5.9 ± 0.1	-9.1 ± 3.6
Glycine	-7.4 ± 0.5	-11.6 ± 1.2
Proline	-4.9 ± 1.0	-24.4 ± 3.0

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).

It would be interesting to investigate the transport upon upshock of the two other compatible solutes, trehalose and ectoine, that are also synthesized by *E.*

Fig. 4. Structure of various compounds used as analogues in glycine betaine transport.

halochloris (Galinski & Herzog, 1990; Peters *et al.*, 1990). It will also be of interest to examine the properties of the transport protein and the molecular regulation of its expression. Detailed studies in *E. coli* (May *et al.*, 1986; Barron *et al.*, 1987) and in *S. typhimurium* (Cairney *et al.*, 1985*a, b*; Sutherland *et al.*, 1986; Higgins *et al.*, 1987) proved the existence of a low-affinity system, ProP, and a high-affinity transport system, ProU, which is induced by high osmolarities. The *proU* gene of these bacteria encodes a periplasmic betaine binding protein with a molecular mass of about 32 kDa (May *et al.*, 1986; Cairney *et al.*, 1985*a*). Only recently, a periplasmic binding protein was identified in *R. meliloti* (Le Rudulier *et al.*, 1991) and *A. brasilense* sp7 (Riou *et al.*, 1991). It remains to be shown whether the ProP and ProU systems are involved in glycine betaine transport in *E. halochloris*.

Structural specificity of glycine betaine transport

The structural specificity of the glycine betaine transport system was tested by examining the effect of various non-radioactive competitors (Table 2). Uptake of radioactive glycine betaine was measured in the presence of competitor concentrations 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. Rowland & 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 $\mu\text{g ml}^{-1}$), which inhibits *de novo* protein synthesis and cell growth, had no effect on uptake, indicating that the transport system is constitutive.

This study was supported by a grant from the German-Israeli Foundation for Scientific Research and Development (GIF). P. Peters gratefully acknowledges the great hospitality extended to her during her stay in Rehovot, Israel. Thanks are due to Wolfgang Hipp for valuable scientific discussions.

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