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Identification of an osmotically induced periplasmic glycine betaine-binding protein from *Rhizobium meliloti*

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The effect of salt stress on glycine betaine-binding activity has been investigated in periplasmic fractions released from *Rhizobium meliloti* 102F34 by cold osmotic shock. Binding activity was monitored by three techniques: equilibrium dialysis, filter procedure, and detection of ^{14}C ligand-protein binding by direct non-denaturing polyacrylamide gel electrophoresis (PAGE) followed by autoradiography. The three methods demonstrated the existence of a strong glycine betaine-binding activity, but only in periplasmic fractions from cells grown at high osmolarity. The non-denaturing PAGE of such periplasmic shock fluids mixed with [*methyl*-^{14}C]glycine betaine showed only one radioactive band, indicating the involvement of one glycine betaine-binding protein. To determine the possible implication of this binding protein in glycine betaine uptake, transport activity was measured with cells submitted to cold osmotic shock. No significant decrease of transport activity was noticed. This lack of effect could be explained by the small quantity of periplasmic proteins released as judged by the low activity of phosphodiesterase, a periplasmic marker enzyme, observed in the shock fluid. The specificity of binding was analysed with different potential competitors: other betaines such as γ-butyrobetaine, proline betaine, pipecolate betaine, trigonelline and homarine, or amino acids like glycine and proline, did not bind to the glycine betaine-binding protein, whereas glycine betaine aldehyde and choline were weak competitors. Optimum pH for binding was around 7.0, but approx. 90% of the glycine betaine-binding activity remained at pH 6.0 or 8.0. The calculated binding affinity (K_D) was 2.5 μM. Both glycine betaine-binding activity and affinity were not significantly modified whether or not the binding assays were done at high osmolarity. A 32 kDa osmotically inducible periplasmic protein, identified by SDS-PAGE, apparently corresponds to the glycine betaine-binding protein.

Introduction

Glycine betaine is one of the most important osmoprotective compatible solutes which can be accumulated to high intracellular concentrations in several groups of Gram-negative eubacteria, including halotolerant bacteria [1], extreme and moderate halophiles [2,3], and nonhalophilic bacteria [4–7]. In *Escherichia coli* and *Salmonella typhimurium*, the uptake of glycine betaine from the external medium is stimulated by high osmolarity and confers high levels of osmotic tolerance [4,5,8]. In these organisms, two genetically distinct transport systems have been identified and studied extensively: a relatively low-affinity transport system encoded by *pro P* which has also affinity for proline [9], and a high-affinity transport system encoded by the *pro U* locus [5,10]. Following osmotic upshock, the expression of *pro P* is increased 3- or 4-fold [9,11], whereas the transcription of *pro U* is enhanced 100-fold [5–12]. The Pro U system of *E. coli* and *S. typhimurium* is binding-protein dependent; a 31 kDa periplasmic glycine betaine-binding protein has been characterized [13,10]. This protein was purified from *E. coli* and was shown to have a K_D of 1.4 μM for glycine betaine [14]. Recently, analysis of the nucleotide sequence of the *pro U* operon of *E. coli* has shown that this locus contains three genes, *pro V*, *pro W* and *pro X* [15,16]. From the determined *pro U* DNA sequence [17], the product of these genes have been deduced. The *pro V* gene encodes

Abbreviations: PDE, cyclic phosphodiesterase; MDH, malate dehydrogenase.

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a hydrophilic protein which shares considerable sequence identity with ATP-binding proteins from other periplasmic transport systems [18,19]. The *pro W* gene encodes a hydrophobic polypeptide which is thought to be located in the cytoplasmic membrane. The *pro X* gene encodes the periplasmic glycine betaine-binding protein. Glycine betaine is also an effective osmoprotectant in *Rhizobium meliloti*, the root nodule symbiont of *Medicago sativa* [20,21]. This bacterium accumulates glycine betaine when cultures are grown at inhibitory osmolarity, salvaging both carbon and nitrogen [21]. Osmotic stress strongly decreases the activities of the enzymes involved in the degradation of glycine betaine [22]; thus, glycine betaine alone did not support growth in media of high osmolarity. As in *E. coli* and *S. typhimurium*, glycine betaine transport in *R. meliloti* is strongly stimulated when the cells are grown in media of elevated osmolarity [21]; similar results have also been reported for proline betaine [23], a betaine produced by alfalfa. However, it is obvious that betaine transport is much less understood in this symbiotic bacterium than in enteric bacteria. The data described below demonstrate the existence of a glycine betaine-binding protein in the periplasm of *R. meliloti* grown at high osmolarity. Identification and binding specificity are also reported. The results are discussed in terms of a possible role in transport for this glycine betaine-binding protein.

Materials and Methods

Chemicals. [*methyl*-^{14}C]Glycine betaine was prepared from [*methyl*-^{14}C]choline (2.15 MBq/μmol, CEA, Gif sur Yvette, France), and purified using paper high-voltage electrophoresis [23]. γ-[U-^{14}C]Butyrobetaine (0.59 MBq/μmol) was kindly provided by J. Deshusses, University of Geneva, Switzerland. Radioactive proline betaine was synthesized from L-[U-^{14}C]proline (9.62 MBq/μmol, CEA, Gif sur Yvette, France) as previously reported [23]. Glycine betaine, glycine betaine aldehyde, choline, trigonelline and amino acids were purchased from Sigma. Homarine, proline betaine, γ-butyrobetaine and pipecolate betaine were obtained as mentioned earlier [4]. All other reagents were of the highest purity available from Merck (Darmstadt, F.R.G.).

Bacterial strains and growth conditions. R. meliloti, strain 102F34, was maintained on solid MSY medium (mannitol-salts-yeast extract [24]). The minimal medium (LAS) contained 0.1% (w/v) D,L-sodium lactate, 0.13% sodium aspartate, 20 μg of biotin per l, and the same salts as in MSY. *S. typhimurium*, strain LT2 (wild-type), and strain CH 946 (mutant defective in the Pro U transport system, [5]) were maintained on solid LB medium [25], and grown on minimal M63 medium [26]. The osmolarity of minimal media was increased by the addition of NaCl. Cultures were grown aerobically with shaking at 200 rpm, at 30°C for *R. meliloti*, and 37°C in the case of *S. typhimurium*. Inocula were grown overnight in MSY or LB medium, and used at a final concentration of 1%. Bacterial growth was monitored spectrophotometrically at 420 nm, and the protein concentration of the bacterial culture was determined as in Ref. 27.

Glycine betaine uptake assays. Individual uptake experiments were performed aerobically as indicated earlier [4,21]. Each assay contained 2.08 kBq of [*methyl*-^{14}C]glycine betaine and nonradioactive glycine betaine to produce the appropriate specific radioactivity. Uptake was terminated by rapid filtration through cellulose-nitrate filter (0.45 μm pore size; Millipore). The filters were washed immediately with 5 ml of the corresponding growth medium, and placed in scintillation vials containing 4 ml of ACS liquid (Amersham). Radioactivity was determined in a Kontron liquid scintillation spectrometer. Each assay was done at least in triplicate, and each experiment was repeated with three independent cell suspensions.

Isolation of periplasmic fractions and binding assays. Cells were harvested by centrifugation (10000 × *g*, 5 min, 30°C) when the culture density reached 1.0 absorbance unit. They were washed and resuspended in 10 mM Tris-HCl (pH 7.5). Periplasmic proteins were released by cold osmotic shock according to Ref. 28. Control cells were treated similarly except that sucrose was omitted. Periplasmic fractions were filtered on a 0.22 μm membrane filter (Millipore), concentrated by ultrafiltration on Amicon YM 10 membranes, and centrifuged 1 h at 100000 × *g* (4°C). Detection of binding activity in shock fluid was performed by three techniques. First, by equilibrium dialysis technique [23,19], at 20°C, in the presence of 10 μM [*methyl*-^{14}C]glycine betaine, [*methyl*-^{14}C]choline, γ-[^{14}C]butyrobetaine, L-[U-^{14}C]proline, or 5 μM [^{14}C]proline betaine. The protein concentration was constant at 0.5 mg/ml, and samples (20 μl) were taken in duplicate every 30 min over 6 h. The amount of radioactive substrate remaining in the dialysis bag was determined by scintillation counting. Second, as an alternative, the binding assay was done by ammonium sulphate precipitation [30]. Samples of 100 to 200 μg protein of periplasmic shock fluid were incubated, at 20°C, for 10 min with various concentrations of ^{14}C substrate (0.5 to 10 μM). Proteins were precipitated by adding 1 ml of ice-cold saturated ammonium sulphate solution. The radioactivity of the filters was determined by scintillation counting. Third, binding activity was detected by direct polyacrylamide gel electrophoresis of the complex ligand-protein in non-denaturing conditions as described in Ref. 31. Complexes of radioactive ligands with periplasmic binding protein are sufficiently stable to be detected by autoradiography after non-denaturing PAGE using a minigel system. Details are given in the following section.

Protein gel electrophoresis and autoradiography. The periplasmic proteins were analysed by polyacrylamide gel electrophoresis using a Bio-Rad mini protean slab cell system (Bio-Rad, Richmond, CA). SDS-PAGE was performed as in Ref. 32, using a 10.5% gel. The protein standards were from Bio-Rad. Non-denaturing PAGE was conducted as follow. Aliquots of the concentrated shock fluids (50 μg) were mixed with the respective radioactive ligands, at a final concentration of 10 μM (except in the case of proline betaine, 5 μM), and left at 20°C for 30 min. In experiments done to test the specificity of the binding, unlabelled competitors (final concentration 1 mM) were added. Laemmli's sample buffer [32] without SDS and β-mercaptoethanol was then added, and the volume of the samples was reduced under vacuum. These samples were subjected to PAGE (15.0% gel) in a discontinuous system in which SDS was omitted. The analyses were performed with a constant voltage (200 V) for approx. 45 min. The gels were quickly dried on Whatmann 3 MM paper and autoradiographied using X-OMAT S Kodak films during 8–10 days. Staining can be done after autoradiography by rapidly scraping the paper from the gel with distilled water. Protein bands were visualized by staining with Coomassie blue or silver [33]. In order to identify the glycine betaine-binding protein, a preparative non-denaturing gel was performed, each well was loaded with the same amount of periplasmic proteins (50 μg). After the run, one lane was rapidly stained to identify the band that binds glycine betaine, and the appropriate area of the unstained gel was cut out. The gel slice was electro-eluted using a model 422 electro-eluter system (Bio-Rad). The eluted proteins were concentrated on microconcentrator (Centricon 10, Amicon), and submitted to SDS-PAGE.

Enzyme assays. The activity of one periplasmic marker enzyme, cyclic phosphodiesterase (also referred to as phosphodiesterase), and the activity of one typical cytoplasmic marker, malate dehydrogenase, were measured to determine the amount of marker enzymes activities released from cells by cold osmotic shock. The assays for phosphodiesterase (PDE) were the same as described previously [34]. For the assay of malate dehydrogenase (MDH), the reaction mixture contained 2.3 ml of 100 mM KH_2PO_4 (pH 7.5), 100 μl of 7.1 mM NADH, and sample. After determination of control ΔA_{340}, 100 μl of 30 mM oxaloacetate was added, and oxaloacetate-dependent ΔA_{340} was taken as enzyme activity. Enzyme activities were measured, in parallel, in cells following cold osmotic shock and submitted to sonication, and in periplasmic shock fluids. For calculation of proportional activity, i.e., activity in periplasmic fractions as a percentage of total activity, activity was expressed as nmol/min for the total samples of cells, equivalent to 60 ml of culture $A_{420} = 1.5$.

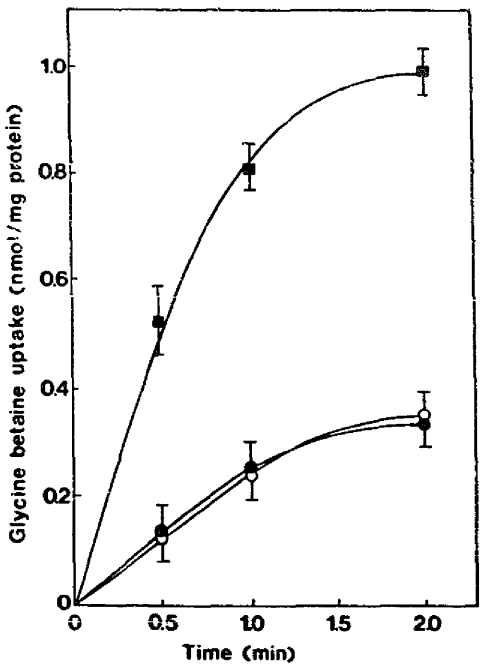

Fig. 1. Time course of glycine betaine uptake by shocked and control cells of *R. meliloti* 102F34. Uptake of 10 μM [*methyl*-^{14}C]glycine betaine was measured in cells treated as described in Materials and Methods. Symbols: ■, untreated cells; ●, osmotic cold-shocked cells; ○, cold shocked cells. Each point is the mean of duplicates carried out on three independent preparations of periplasmic fractions.

Results

Sensitivity of glycine betaine uptake to cold osmotic shock

Because uptake systems which involve a specific periplasmic binding protein are well known to be inhibited by cold osmotic shock, *R. meliloti* cells grown in LAS medium of high osmolarity (0.3 M NaCl) were submitted to this procedure. Glycine betaine uptake was then measured and compared to that of non osmotically shocked cells (Fig. 1). The cold shock significantly inhibited the transport activity (64%), whereas the cold osmotic shock did not have any additional effect. This pointed out the necessity of this control, which is often omitted. At the same time, a 20% decrease of the viability of the cells was observed after the cold shock, but the cold osmotic shock had approximately the same consequence.

The lack of inhibitory effect of osmotic shock on glycine betaine uptake might be due to unsuccessful in releasing more than a small fraction of periplasmic proteins. To test this hypothesis, activity of one periplasmic marker enzyme was measured in shock fluids and in sonicated cells (Table I). The cold osmotic shock

resulted in the release of only 1.2% of the PDE activity which was measured following sonication of the cells. Absolutely no MDH activity was observed in shock fluids, whereas this activity was very high in the sonicated fraction (Table I). This enzyme is a very useful cytoplasmic marker, so that even very small losses due to moderate alterations of cytoplasmic membrane integrity could be accurately measured. The difficult release of periplasmic proteins was not specifically observed with the strain 102F34, since similar results were obtained with *R. meliloti* 2011 (data not shown). However, differences have been noticed between bacterial species. For example, with *Bradyrhizobium japonicum* only 10% of the total PDE activity could be released by osmotic shock, whereas 50% was measured with *R. phaseoli* [34].

Evidence for glycine betaine-binding activity in periplasmic fractions

Although only a small fraction of periplasmic proteins could be released by osmotic cold shock, it was of interest to determine whether released proteins might function as binding proteins for glycine betaine. In a first attempt, the dialysis technique based on the retention phenomenon of binding proteins [35] was used. Fig. 2 shows a rapid and linear exit of free radioactive glycine betaine from a dialysis bag that contained only buffer or periplasmic shock fluid from cells grown at low osmolarity. The half-life of release of substrate was 16 min. On the contrary, with concentrated shock fluid from cells grown at high osmolarity, the initial rapid efflux of excess glycine betaine was followed by a slow dissociation of bound substrate. After 120 min, the half-life of release increased to 120 min. In similar experiments the rate of release of [*methyl*-^{14}C]choline, γ-[^{14}C]butyrobetaine, [^{14}C]proline betaine or [^{14}C]proline was not significantly reduced by the presence of periplasmic proteins obtained from 0.3 M NaCl-grown cells. Furthermore, addition of unlabelled choline, γ-butyrobetaine, proline betaine or proline (1 mM final concentration), used as potential competitors in the dialysis bag, did not modified the release of [*methyl*-^{14}C]glycine betaine. On the contrary, addition of unlabelled glycine betaine gave a 18 min half-life of release of [*methyl*-^{14}C]glycine betaine, a value similar to that obtained in the absence of proteins. Hence, binding of glycine betaine is likely to be specific. Dialysis experiments done with periplasmic proteins extracted from *S. typhimurium* LT2 cells grown at high osmolarity (0.3 M NaCl) have confirmed the existence of glycine betaine binding activity [10,13], but have also demonstrated binding for [^{14}C]proline betaine. Binding activity for both betaines totally disappeared when shock fluids from strain CH 946 (mutant defective in the Pro U transport system) were used (data not shown).

In a second approach, the substrate-binding affinity for glycine betaine was determined using ammonium sulphate precipitation [30,13]. Binding activity at different substrate concentrations was assayed in periplasmic fractions isolated from *R. meliloti* cells grown at high osmolarity (Fig. 3). Assuming that one molecule of protein binds one molecule of glycine betaine, the calculated K_D was approx. 2.5 μM, which is in good

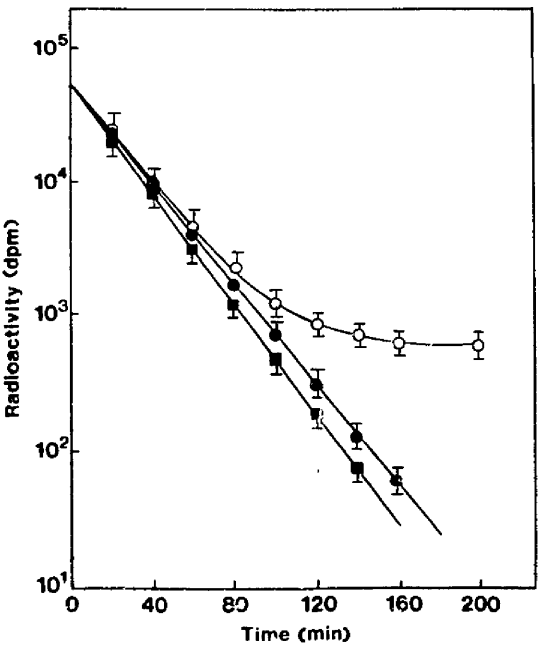

Fig. 2. Retention of glycine betaine by periplasmic shock fluid of *R. meliloti* 102F34. Binding of [*methyl*-^{14}C]glycine betaine (10 μM) was measured with periplasmic proteins (0.5 mg/ml) from low salt-grown cells (●), and high-salt-grown cells (○). Binding was assayed at 10 mM Tris-HCl (pH 7.5). The symbol (■) indicates a control experiment without periplasmic proteins in the assay. Each point is the mean of results from at least five independent experiments.

TABLE I

Release of marker enzymes from R. meliloti 102F34 following cold osmotic shock

Bacteria were grown at high osmolarity (0.3 M NaCl), and harvested when the culture density reached an $A_{420} \approx 1.5$. The cold osmotic shock, and enzyme assays were performed as described in Materials and Methods.

Fraction assayed	Phosphodiesterase		Malate dehydrogenase	
	activity [a]	% of total	activity [a]	% of total
Periplasm	0.6	1.2	< 5	< 0.1
Sonicated cells	48.8	98.8	7395	100
Total	49.4	100	7395	100

[a] Units are nmol/min for the total sample of cells, equivalent to 60 ml of culture.

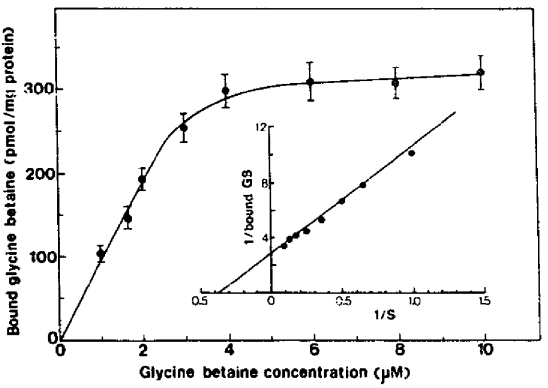

Fig. 3. Glycine betaine-binding activity in periplasmic shock fluid of *R. meliloti* 102F34. Periplasmic proteins were isolated from cells grown at high osmolarity (NaCl 0.3 M), and binding activity was assayed using ammonium sulphate precipitation at various glycine betaine concentrations in 10 mM Tris-HCl (pH 7.5). Each assay corresponded to 200 μg protein. Each point is the mean of duplicates carried out on three independent preparations of periplasmic fractions.

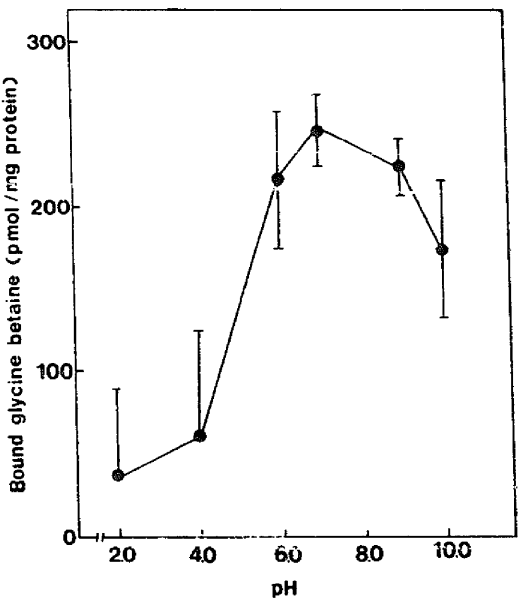

Fig. 4. Effect of pH on glycine betaine-binding activity in periplasmic shock fluid of *R. meliloti* 102F34. Binding of [*methyl*-^{14}C]glycine betaine was assayed as in Fig. 3, with 10 μM glycine betaine. Each point is the mean of duplicates carried out on two independent preparations of periplasmic fractions.

agreement with K_t previously measured (4.3 μM) for glycine betaine transport in *R. meliloti* [36]. The maximal capacity of binding was 310 pmol/mg of periplasmic protein with a free ligand concentration of 9 μM. With periplasmic fractions from cells grown at low osmolarity, the binding activity was less than 65 pmol/mg protein. Since the amount of periplasmic proteins released is always approx. 2.5-times lower with low osmolarity-grown cells than with high-osmolarity-grown cells, we can estimate that the glycine betaine binding capacity of 1 l of cell suspension ($A_{420} = 1$) is about 12-times higher in cells subjected to salt stress (155 ± 17 pmol) than in control cells (13 ± 2 pmol).

TABLE II

Effect of osmolarity in the growth medium, and osmotic strength in the binding assay on glycine betaine binding activity of periplasmic shock fluid from R. meliloti 102F34

Bacteria were grown at low osmolarity (LAS medium) or high osmolarity (LAS with 0.3 M NaCl), and harvested at $A_{420} = 1.5$. Periplasmic fractions were prepared as described in Materials and Methods. Binding assays were done in 10 mM Tris-HCl (pH 7.5) with NaCl added as indicated, and saturating glycine betaine concentration (10 μM). Each assay corresponded to 200 μg protein, and each value is the mean of duplicates carried out on three independent preparations of periplasmic fractions.

Osmolarity of growth medium	NaCl added to binding assay	Glycine betaine bound (pmol/mg protein)
Low	none	65 ± 5
Low	0.3 M	62 ± 5
High	none	290 ± 40
High	0.15 M	280 ± 36
High	0.3 M	295 ± 42

Binding activity of periplasmic shock fluids from low- and high-osmolarity-grown cells was also determined at various osmotic strength (Table II). With shock fluids of both origins, glycine betaine-binding was found to be identical, whether or not NaCl was mixed to the assay. Similarly, the K_D was not significantly modified (data not shown). Thus, binding was not stimulated or inhibited by high osmotic strength.

The pH dependence of the binding activity was assayed with periplasmic proteins isolated from cells grown at high osmolarity, using ammonium sulphate precipitation. Optimum pH for binding was approx. 7.0 and the binding activity was not much affected between pH 6.0 and 8.0 (Fig. 4).

Non-denaturing polyacrylamide gel electrophoresis

Crude shock fluids from *R. meliloti* cells grown at high or low osmolarity were incubated with [*methyl*-^{14}C]glycine betaine as described in Materials and Methods, and subjected to non-denaturing PAGE and autoradiography (Fig. 5). The most striking feature of salt stress was a decrease in the abundance of proteins with relatively low charge, whereas the abundance of highly negative charged proteins was rather increased. On the autoradiogram, only one radioactive band was found when the cells were grown in the presence of high salt, showing the presence of the label bound to one binding protein. On the contrary, shock fluid from cells grown

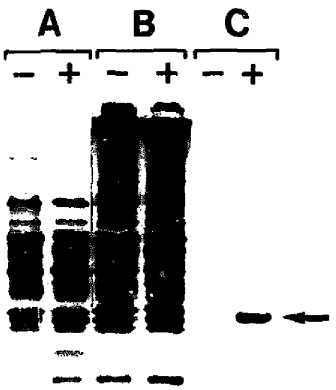

Fig. 5. Non-denaturing polyacrylamide gel, 15% (A and B), and autoradiography (C) of periplasmic proteins from *R. meliloti* 102F34. Proteins were released by cold osmotic shock from cells grown in minimal medium (−), and minimal medium with 0.3 M NaCl (+). The gels were stained with Coomassie blue (A, 50 μg protein), and silver-stain method (B, 5 μg protein). A 50 μg amount of the proteins was incubated with 10 μM [*methyl*-^{14}C]glycine betaine, subjected to electrophoresis and then autoradiographed. The arrow corresponds to the position of the glycine betaine-binding protein.

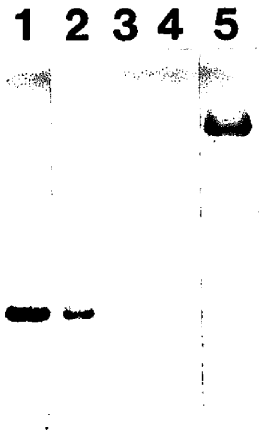

Fig. 6. Autoradiography of periplasmic proteins from *R. meliloti* 102F34 (lanes 1 to 4) and from *S. typhimurium* strain LT2 (lane 5), subjected to 15% non-denaturing polyacrylamide gel electrophoresis. Proteins were released by cold osmotic shock from cells grown in minimal medium with 0.3 M NaCl. A 50 μg amount of the proteins was incubated with 10 μM [*methyl*-^{14}C]glycine betaine (lanes 1 and 5), 10 μM [*methyl*-^{14}C]choline (lane 2), 10 μM γ-[^{14}C]butyrobetaine (lane 3), and 5 μM [^{14}C]proline betaine (lane 4).

in the absence of salt did not show any label bound. It is clear, using Coomassie blue or silver staining, that the amount of proteins found at the same position as the radioactive band, was much higher in cells grown at high osmolarity than in cells maintained in low salt medium. This band probably corresponds to several proteins, and we can suggest that one of these proteins, absent or in very low amount in control cells and well represented in high osmolarity-grown cells, serves as a glycine betaine-binding protein.

The specificity of the binding was assayed by two different ways. First, periplasmic proteins from 0.3 M NaCl-grown cells were incubated with [*methyl*-^{14}C] glycine betaine as a control, [*methyl*-^{14}C]choline, γ-[^{14}C]butyrobetaine or [^{14}C]proline betaine (Fig. 6). With [^{14}C]choline, a weak labelling was observed at the same position as the strong radioactive band obtained with [^{14}C]glycine betaine, whereas absolutely no binding could be seen with other betaines. However, in the presence of γ-[^{14}C]butyrobetaine, a very feeble band which corresponds to a different protein appeared. One can suggest that the difference in the intensity of the labelling could be related to different specific radioactivities of the ligand. This is unlikely, because highest specific activity was observed in [^{14}C]proline betaine which did not bind, and [^{14}C]choline and [^{14}C]glycine betaine have the same specific activity. In addition, γ-[^{14}C]butyrobetaine, which has a relatively low specific activity, gave a strong labelling with a 52 kDa periplasmic protein from *Agrobacterium* [31]. Incubating periplasmic shock fluid from *S. typhimurium* LT 2 cells grown at 0.3 M NaCl with [*methyl*-^{14}C]glycine betaine showed that the glycine betaine-binding protein characterised in this bacterium [13] has a much less negative entire charge than the binding protein from *R. meliloti*. In a second approach, periplasmic shock fluid fractions from *R. meliloti* cells grown at 0.3 M NaCl were incubated with various unlabelled potential competitors (1 mM final), before the addition of [*methyl*-^{14}C]glycine betaine (10 μM final). When unlabelled glycine betaine was added, the labelling totally disappeared, demonstrating the specificity of the binding phenomenon (Fig. 7, lane 2). Addition of glycine betaine aldehyde significantly decreased the labelling, and addition of choline seemed, to a lesser extent, to have the same effect. On

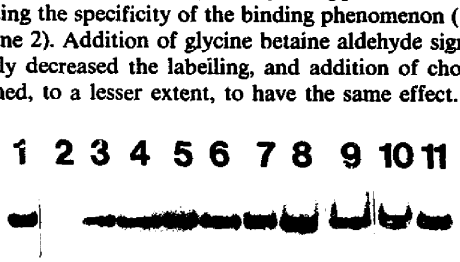

Fig. 7. Autoradiography of periplasmic proteins from *R. meliloti*, 102F34 subjected to 15% non-denaturing polyacrylamide gel electrophoresis. Proteins were released by cold osmotic shock from cells grown in minimal medium with 0.3 M NaCl. A 50 μg amount of the proteins was incubated with 10 μM [*methyl*-^{14}C]glycine betaine in the absence (lane 1) or the presence (lane 2) of 1 mM unlabelled glycine betaine. Lanes 3 to 11 contained the same amount of periplasmic proteins (50 μg), 10 μM [*methyl*-^{14}C] glycine betaine, and 1 mM of unlabelled betaines or analogs: glycine betaine aldehyde, choline, γ-butyrobetaine, glycine, proline, proline betaine, pipecolate betaine, trigonelline or homarine, respectively from lane 3 to lane 11.

Fig. 8. SDS-PAGE (10.5%) of the electro-eluted periplasmic proteins from *R. meliloti* 102F34. A preparative non-denaturing gel was performed, the band that binds glycine betaine was cut out, and electro-eluted. Proteins were from cells grown at high osmolarity (lane 2), or low osmolarity (lane 3). The gel was stained with Coomassie blue. The glycine betaine-binding protein (GBBP) is indicated, as is the mobility of molecular mass markers (lane 1).

the contrary, the presence of other betaines (γ-butyrobetaine, proline betaine, pipecolate betaine, trigonelline or homarine), or amino acids (glycine or proline), had no effect on the intensity of the radioactive band. Hence, we confirm that the specificity of the binding phenomenon is very narrow.

Identification of the glycine betaine-binding protein: SDS-PAGE

Because of the existence of a strong glycine betaine-binding activity in cells grown in the presence of salt, and the absence of labelling in the corresponding band on non-denaturing gel when the cells were grown at low osmolarity, we decided to examine on a SDS-gel the profile of proteins electro-eluted from this zone (Fig. 8). As predicted, several proteins were present in periplasmic fractions of both origines. Due to the difficulty of cutting out only the narrow band which corresponds to the complex ligand-protein on the non-denaturing gel, it might be possible that some proteins observed on the SDS-gel came from the close surrounding of the radioactive zone. However, a similar pattern was observed with protein from cells grown at high or low osmolarity, except for one protein which was fairly abundant in the first growth condition and virtually absent in the second (Fig. 8, lanes 2 and 3). This osmotically inducible periplasmic protein with an apparent molecular weight of 32000 is apparently the glycine betaine-binding protein. To test the validity of our method, periplasmic fractions from *S. typhimurium* LT2 cells grown at high or low osmolarity were subjected to the same protocol (i.e., non-denaturing gel, electro-elution of the binding band and SDS-PAGE). As expected from previous results [13,41], the 31 kDa glycine betaine-binding protein was well represented at high osmolarity and virtually absent at low osmolarity (data not shown).

Discussion

The results obtained in this study demonstrate the presence of a glycine betaine-binding protein in periplasmic shock fluid of *R. meliloti* cells grown at high osmolarity. The complex of [*methyl*-^{14}C]glycine betaine with the binding protein is sufficiently stable to be detected by autoradiography after non-denaturing polyacrylamide gel electrophoresis (Fig. 5), as shown previously for γ-butyrobetaine-binding protein in *Agrobacterium* sp. [31]. The dissociation constant for glycine betaine, 2.5 μM, is in good correspondence with those reported for other periplasmic binding proteins, between 0.1 and 1.3 μM for sugar substrates [37,38], around 0.1 μM for aminoacids [39], and close to 1 μM for glycine betaine [10,13,14] or γ-butyrobetaine [40]. Modifications of the ionic strength in the binding assay do not modulate the glycine betaine-binding activity (Table II), as previously reported for periplasmic fractions from *E. coli* [10] and *S. typhimurium* [13]. The binding activity is highly specific for glycine betaine, among analogous compounds only glycine betaine aldehyde and choline slightly affect the protein-substrate recognition process. Other betaines, aliphatic such as γ-butyrobetaine, or aromatic like proline betaine, pipecolate betaine, trigonelline and homarine are totally inefficient. In addition, glycine and proline do not display any competitive activity (Fig. 7). Thus, the *N*-trimethyl group seems to play a crucial role in binding to the protein. This specificity is not so narrow in *S. typhimurium*. Indeed, with periplasmic fractions of wild-type cells grown at high osmolarity, we have detected a significant binding activity for [^{14}C]proline betaine in addition to that observed for glycine betaine. Binding activity for both betaines was totally absent in shock fluids prepared from the mutant defective in the Pro U system (strain CH 946). Taken together with the strong reduction in transport activity for both betaines in this mutant, the results suggest that proline betaine is also transported by the high-affinity system encoded by the *pro U* region in *S. typhimurium*.

In *R. meliloti*, the periplasmic protein which binds glycine betaine has an apparent molecular weight of 32000, a value in close agreement with the results obtained in *E. coli* [10,12,14–16] and *S. typhimurium* [13,18,41]. Interestingly, this protein is much more acidic

than the glycine betaine-binding protein from *S. typhimurium* (Fig. 6). In *Agrobacterium*, the γ-butyrobetaine-binding protein (52 kDa) has also been characterised as a very acidic protein with a low isoelectric point (4.3), and a high content of aspartate and glutamate.

Whether or not the glycine betaine-binding protein is involved in transport activity in *R. meliloti* remains an open question. The absence of reduction of glycine betaine transport activity in cells subjected to cold osmotic shock (Fig. 1) mimics a shock-resistant system. However, this lack of effect could certainly be explained by the difficulty in releasing substantial amount of periplasmic proteins. Indeed, the activity of phosphodiesterase, a periplasmic marker enzyme, was always very low in shock fluid compared to the total activity obtained after sonication of the cells (Table I). On the contrary, the good correspondence between the dissociation constant, 2.5 μM, and the previous reported K_t, 5 μM, for glycine betaine transport in *R. meliloti* [36], together with properties very similar to those of glycine betaine-binding protein from enteric bacteria [10,13,14], are indicative of the possible implication of the glycine betaine-binding protein in the transport process in the root nodule symbiont. Another argument for this role comes from preliminary assays with vesicles obtained from cytoplasmic membrane lacking periplasmic proteins; using such vesicles transport activity could not be detected (unpublished data). Although it might be difficult to prepare large amount of purified glycine betaine-binding protein from *R. meliloti*, reconstitution of binding protein-dependent transport process in vesicles could be considered. On the other hand, periplasmic proteins are also well characterised as essential components involved in chemotaxis by acting as initial receptor; among these are the *E. coli* galactose- and maltose-binding proteins [42,43]. At present, we cannot rule out the possibility for the identified glycine betaine-binding protein to be involved in chemotaxis. Only a genetic analysis of the glycine betaine transport system, currently under way, will clearly allow us to determine a real connection between the binding protein and glycine betaine transport in *R. meliloti*.

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