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Characterization of a Periplasmic Protein Related to sn-Glycerol-3-Phosphate Transport in Escherichia Coli

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The cold osmotic shock procedure releases a protein (GLPT) from the cell envelope of Escherichia coli that is related to the transport of sn-glycerol-3-phosphate in this organism. The evidence for this correlation is as follows: 1) GLPT is under the regulatory control of the glpR gene. 2) Some glpT mutants that were isolated as phosphonomycin resistant clones do not synthesize GLPT. Revertants of these mutants (growth on sn-glycerol 3-phosphate) again synthesize GLPT. 3) Some amber mutations in glpT reduce the amount of GLPT while suppressed strains produce normal amounts. 4) Transfer of a plasmid carrying the glpT genes into a strain lacking GLPT and sn-glycerol-3-phosphate transport restores both functions in the recipient. Transport and GLPT synthesis in the plasmid carrying strain are increased 2- to 3-fold over a fully induced wild-type strain, but appear to be constitutive. GLPT is a soluble protein of molecular weight 160,000 composed of 4 identical subunits. The 160,000 molecular weight complex is stable in 1% sodium dodecylsulfate at room temperature. Upon boiling in 1% sodium dodecylsulfate GLPT dissociates into its subunits. Likewise, 8 M urea at room temperature dissociates GLPT into its subunits. Dialysis of dissociated GLPT against phosphate or Tris-HCl buffer, pH 7.0, allows renaturation to the tetrameric form. The protein is acidic in nature (isoelectric point 4.4).

In contrast to the typical transport-related periplasmic-binding proteins, no conditions could be found where pure GLPT exhibited binding activity toward its supposed substrate, sn-glycerol-3-phosphate.

In vivo new appearance of transport activity for sn-glycerol-3-phosphate transport occurs only shortly before cell division. However, GLPT synthesis does not fluctuate during the cell cycle. The available evidence indicates a cell-division-dependent processing of GLPT in the cell envelope as a reason for the alteration in transport activity.

Transport in whole cells is sensitive to the cold osmotic shock procedure, demonstrating the participation of an essential periplasmic component. However, isolated membrane vesicles that are devoid of periplasmic components, including GLPT, are fully active in sn-glycerol-3-phosphate transport. Therefore, we conclude that GLPT is essential in overcoming a diffusion barrier for sn-glycerol-3-phosphate established by the outer membrane. Attempts to isolate mutants that are transport negative in whole

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cells due to a defect in GLPT but are active in isolated membrane vesicles have failed so far. All GLPT mutants tested, whether or not they synthesize GLPT, are not active in isolated membrane vesicles.

Iodination of whole cells with [^{125}I] followed by osmotic shock reveals that several shock-releasable proteins including GLPT become radioactively labeled. This indicates that some portions of GLPT are accessible to the external medium.

Key words: periplasmic proteins, transport, precursor

Recently, the application of a sensitive 2-dimensional polyacrylamide gel electrophoresis technique for the analysis of shock-releasable proteins from E. coli revealed the existence of a protein (GLPT) that was closely related to the transport system of sn-glycerol-3-phosphate (G3P) (Silhavy et al., 1976a).

This transport system previously has been studied mainly by Lin and his collaborators (Lin, 1976) with the following results: 1) Transport of G3P occurs against the concentration gradient without chemical alteration of the substrate. 2) The apparent K_m of the transport system is 12 μM and in E. coli this system is the only one that transports G3P. 3) The transport system is under negative control of the gene product of *glpR*, the regulator gene of the *glp* regulon. 4) The *glpT* mutants that exhibit a phenotypically negative transport behavior for G3P were mapped to be located at min 48 on the linkage map of E. coli, cotransducible with the *nalA* marker. From these studies it was not clear whether or not the *glpT* operon consists of more than one gene.

In the biochemical studies of different bacterial active transport systems, so far 3 types of proteins have been implicated.

A. The M-Protein, the Gene Product of the *lacY* Gene

The M-protein is tightly bound to the inner membrane of E. coli and is most likely the only protein sufficient to account for all the phenomena observed with the lactose transport system (Kennedy, 1970). It is characteristic for this system that it still operates in isolated membrane vesicles (Kaback, 1972) and derives its energy from the proton motive force as defined by Mitchell (Ramos et al., 1976). Several other systems for sugars and amino acids have been found to operate in membrane vesicles. However, their responsible carriers have not been identified.

B. Numerous Soluble Periplasmic Substrate-Binding Proteins

Even though not proven for all binding proteins the following generalization can be made about the soluble periplasmic substrate-binding proteins (Boos, 1974): 1) They establish the recognition site of the respective active transport system. 2) They are not the only component of the system. 3) Binding-protein-mediated transport systems do not operate in membrane vesicles, and their energy coupling may involve the direct participation of cellular ATP (Berger and Heppel, 1974).

C. Outer Membrane Components

These proteins have been identified as transport components that facilitate the diffusion of substrate through the outer membrane. These outer membrane components

usually have additional functions as colicin or phage receptors. For instance, the outer membrane component for the maltose transport system is the receptor for phage λ (Szmelcman and Hofnung, 1975).

The present publication is concerned with the characterization of a shock releasable protein that belongs to the *glpT* operon and may be part of the G3P transport system.

MATERIAL AND METHODS

Bacterial strains used are listed in Table I together with their source. The isolation of phosphonomycin resistant strains was done according to Venkateswasan and Wu (1972). Strains carrying the $glpT^+$ operon on the Col E1 plasmid were obtained by cross-streaking strain JA 200/3-46 with a *glpT* strain on an agar plate containing sn-glycerol-3-phosphate as the sole carbon source. Minimal medium A (Miller 1972) with 0.4% glycerol was used as growth medium. For BUG-6 low-phosphate medium (Garen and Levinthal, 1960) was used with 0.4% glycerol as carbon source. The osmotic shock procedure was done according to Neu and Heppel (1965). Two-dimensional polyacrylamide gel electrophoresis was done according to Johnson et al. (1975) with the modification described by Silhavy et al. (1976a). Purification of GLPT, enzymatic tests, polyacrylmide gel electrophoresis in sodium dodecylsulfate (SDS), and cross-linking of GLPT were described previously (Boos et al., 1977). Preparation of membrane vesicles was done according to Hirata et al. (1974), and transport in these vesicles was measured as described by Boos et al. (1977). Iodination of intact bacteria was done according to Mueller and Morrison (1974). The iodinated cells were subjected to the osmotic shock procedure of Neu and Heppel (1965) and the periplasmic proteins subjected to 2-dimensional polyacrylamide gel electrophoresis. The gels were stained with Coomassie Blue. All spots were cut out, and the gel pieces were solubilized by the addition of 0.5 ml 30% H_2O_2 containing 10% concentrated ammonia. These mixtures were incubated overnight at 42°C. The radioactivity was measured in a liquid scintillation counter after addition of 10 ml dioxan-based scintillation fluid.

To isolate and solubilize the proteins of the cytoplasmic membrane, the exclusive solubilization of these proteins in Triton X-100 in the presence of $MgCl_2$ was used. The procedure was essentially that described by Schnaitman (1971). The solubilized proteins were precipitated in 80% ethanol at 4°C. The precipitate was washed once with 80% ethanol, solubilized in guanidinium thiocyanate, and subjected to 2-dimensional polyacrylamide gel electrophoresis as described by Johnson et al. (1975).

Synchronization technique: Strain 72 was synchronized by a stationary-phase method (Cutler and Evans, 1966) which takes advantage of the tendency of cells to synchronize themselves when they enter the stationary phase of growth.

Transport of sn-glycerol-3-phosphate during synchronous growth: To 100 μl of cells 10 μl of [^{14}C] G3P was added to a final concentration of 1 μM. After 30 sec 100 μl were removed and filtered through a Millipore filter of 0.65 μm pore size. The filter was washed with 10 ml growth medium. This test is linear for bacterial cultures with optical densities (ODs) (at 576 nm) from 0.1 to 1.0. The activities during synchronous growth were expressed as the amount of G3P uptake during the initial 30 sec incubation period.

GLPT pulse labeling: To aliquots of 5 ml of the growing culture [U-^{14}C]-leucine ($0.75 \cdot 10^6$ cpm) and [U-^{14}C]-alanine ($0.75 \cdot 10^6$ cpm) were added at a final concentration of 0.1 μM. Labeling was allowed to proceed for 10 minutes. It was stopped by the addition of an 1,000-fold excess of unlabeled amino acids. The culture was allowed to grow for 10 more minutes. Then, the cells were harvested by centrifugation and washed

TABLE I. Strains of E. Coli Used

Strain no.	Parent	Sex	Isolation procedure	Genotype	Reference
72		Hfr		Δ (*glpR-malA*), *phoA*	Cozzarelli et al. (1968)
LA 3400	72	Hfr	P1 transduction to *mal*$^+$	*glpR*$^+$, *mal*$^+$, *phoA*	Silhavy et al. (1976a)
LA 3404	72	Hfr	Resistance against phosphonomycin, spontaneous mutants	Δ (*glpR-malA*), *phoA*, *glpT*	Silhavy et al. (1976a)
LA 108	BUG-6 temperature sensitive for cell division, i.e., septum formation	F	Spontaneous; growth on β-glycerolphosphate	*strA*, *gal*, *xgl*, *mtl*, *phoR*	Reeve et al. (1970) Shen and Boos (1973)
JA 200/3-46	–	F$^+$		Δ *trpE5*, *recA*; carrying plasmid colE1, pLCNr. 3–46	Clarke and Carbon (1976)
G 810	MC 4/00	F$^-$	Mu cts: :*malT*; P1 transduction to *mal*$^+$, *glpR*; Mu cts: :*glpT*, phosphonomycin resistant	*araD*, Δ*lac*, *strA*, *glpR*, Mu cts::*glpT*	Silhavy et al. (1976b)
G 810/3	G 810	F$^-$	Growth at 42°C	*araD*, Δ*lac*, *strA*, *glpR*, *glpT*	this study
G 810/3 3-46	G 810/3 and JA 200/3-46	F$^+$	Cross-streaking on sn-glycerol-3-phosphate	*araD*, Δ*lac*, *strA*, *glpR*, *glpT*$^+$	this study
165	–	–		*glpR*, *glpD*, *glpA*$^+$, *thr*, *leu*, *thi*	Miki, Harvard Medical school, personal communication

once with growth medium minus carbon source. The cells were then transformed into spheroplasts by the EDTA-lysozyme treatment in the presence of 20% sucrose (Neu and Heppel, 1964). The supernatant of these spheroplasts that contained the proteins of the periplasmic space was analyzed by SDS polyacrylamide gel electrophoresis together with an unlabeled sample of the osmotic shock proteins. The Coomassie-Blue stained band identified on these gels with GLPT was cut from the gel, dissolved in 0.5 ml 30% H_2O_2 containing 50 μl conc. NH_3 and counted in the liquid scintillation counter with 5 ml dioxan-naphtaline based scintillation fluid.

RESULTS

1. Evidence for GLPT Being a Gene Product of the *glpT* Operon

A. Genetics and osmotic shock. Figure 1 shows the 2-dimensional electrophoresis analysis of the shock-releasable proteins from a strain with a fully induced G3P transport

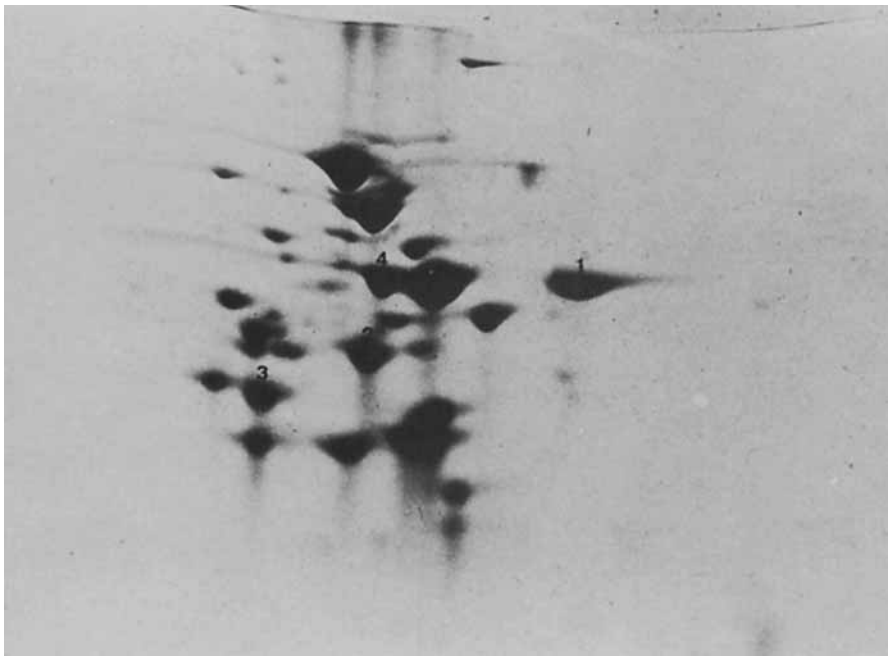

Fig. 1. Two-dimensional polyacrylamide gel electrophoresis of shock fluid of strain LA 3400 (wild-type) grown in the presence of glycerol. The first dimension consists of electrophoresis in 8 M urea (pH 8.4), followed by electrophoresis in 0.2% sodium dodecylsulfate (pH 6.48). About 300 μg of protein was applied. The numbers and molecular weights correspond to the following proteins: 1) GLPT: 2) galactose-binding protein (Boos and Gordon, 1971); 3) ribose-binding protein (Willis and Furlong, 1974); 4) maltose-binding protein (Kellerman and Szmelcman, 1974). With modifications taken from Silhavy et al. (1976a) (with permission from the authors and publisher).

system. Using this technique we previously demonstrated that one spot designated GLPT only appeared in glycerol-grown wild-type cells, or independent of inducer, in *glpR* strains that are constitutive for all *glp* operons. Moreover, some *glpT* mutants that are unable to accumulate G3P lack GLPT, while their revertants again produce it. Treatment of the cells with the cold osmotic shock procedure that releases GLPT results in a drastic reduction of the ability of shocked cells to accumulate G3P (Shilhavy et al., 1976a).

B. Nonsense mutants in *glpT*. The shock-releasable proteins of 3 characterized mutants that carry nonsense mutations in *glpT* and their respective suppressed derivatives (obtained from Dr. Weissenbach, Harvard Medical School, Boston) gave the following picture: 2 mutants synthesized a sharply reduced amount of GLPT, while the suppressed strain again synthesized it. Transport activity for G3P corresponded to the amount of GLPT seen in these mutants. One mutant showed normal GLPT levels, even though it was transport negative. Thus, in 2 of these mutants the nonsense mutation must have occurred in a gene proximal to the gene that codes for GLPT, affecting the amount but not the structure of GLPT. This demonstrates that the *glpT* operon must consist of more than one gene.

C. Insertions of phage Mu into *glpT*. Insertion of phage Mu into any gene of the E. coli chromosome destroys the gene product of this gene and has a strong polar effect on the distal genes of the same operon. Mutants carrying a temperature-sensitive Mu phage in *glpT* were obtained by Dr. Silhavy (Harvard Medical School, Boston) and analyzed for their content of shock-releasable GLPT. Out of 8 transport negative mutants 7 lacked GLPT while 1 contained the normal amount (not shown). This again suggests that GLPT

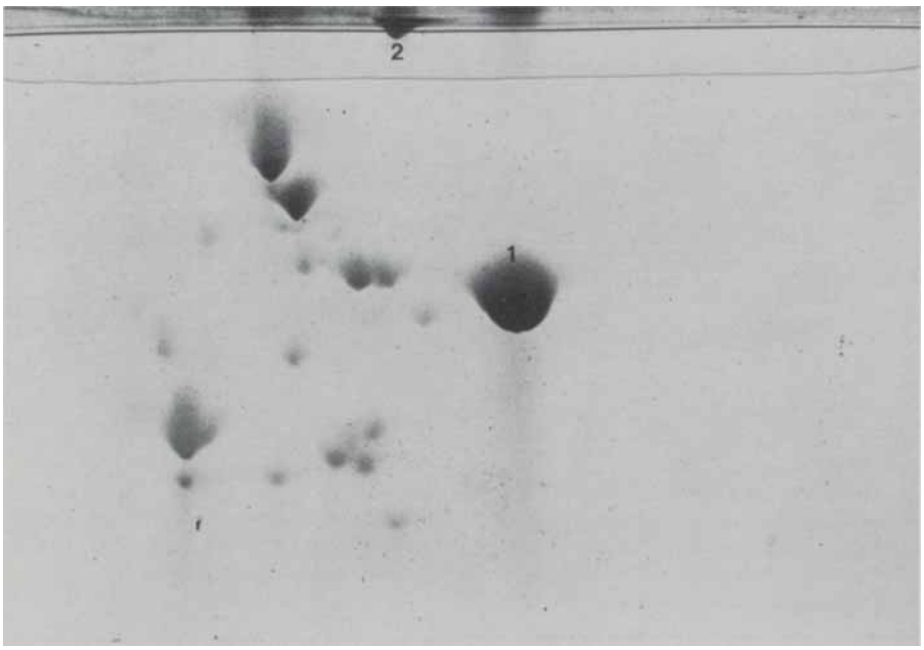

Fig. 2. Two-dimensional polyacrylamide gel electrophoresis of shock fluids of strain JA 200/3-46 grown on glycerol. Spot No. 1 is monomeric GLPT, and No. 2 tetrameric GLPT. Conditions as described in the legend to Fig. 1.

is coded for by a gene that is contained in the *glpT* operon.

D. The *glpT* plasmid. From a bank of colE1 plasmid carrying different genes of the E. coli chromosome (Clarke and Carbon, 1976) 3 strains were obtained containing the *glpT* gene. The electrophoretic analysis of the periplasmic shock proteins of such a strain grown on glycerol is shown in Fig. 2. As can be seen this strain contains a large amount of GLPT. One of the *glpT* carrying plasmids was then transferred into a strain that was transport negative for G3P and did not contain GLPT. Figure 3 shows the transport activity of the recipient strain with and without the plasmid. As a control, the transport activity of a fully-induced normal wild-type strain is included. The introduction of the *glpT* carrying protein results in a 2- to 3-fold increase of transport activity for G3P over a fully-induced wild-type strain.

However, transport activity as well as the amount of GLPT synthesized is not any longer inducible by G3P as is the normal wild-type strain. The GLPT operon of the plasmid is apparently constitutive even though the recipient strain contains a wild-type *glpR* gene product, the regulator protein for the *glp* operon. Figure 4 shows the electrophoretic analysis of the shock proteins of the receptor strain with and without the plasmid. As can be seen the only new spot in the protein pattern is GLPT. The increased transport activity for G3P correlates with the increased amount of GLPT produced by these strains. The size of the *glpT* carrying plasmid was measured as $9-10 \cdot 10^6$ daltons, while the original plasmid DNA is in the order of $4 \cdot 10^6$ daltons. Thus, a DNA segment of $5 \cdot 10^6$ daltons of the E. coli chromosome corresponding to no more than 5 genes is responsible for the transport activity of G3P and the synthesis of GLPT.

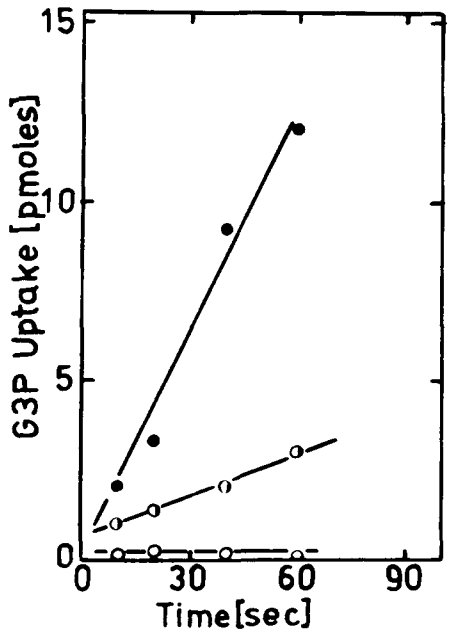

Fig. 3. Transport of sn-glycerol-3-phosphate in *glpT* mutant G 810/3 (○), its *glpT*$^+$ (plasmid) carrying derivative (●), and a normal wild-type strain, LA-3400 (◑). The bacterial cultures were resuspended in 10 mM Tris-HCl, 150 mM sodium chloride (pH 6.5) to an optical density (OD) of 1.0 at 576 nm. [^{14}C]-G3P was added at an initial concentration of 0.3 μM. The data are expressed in terms of amount substrate taken up per 50 μl sample. All operations were done at room temperature.

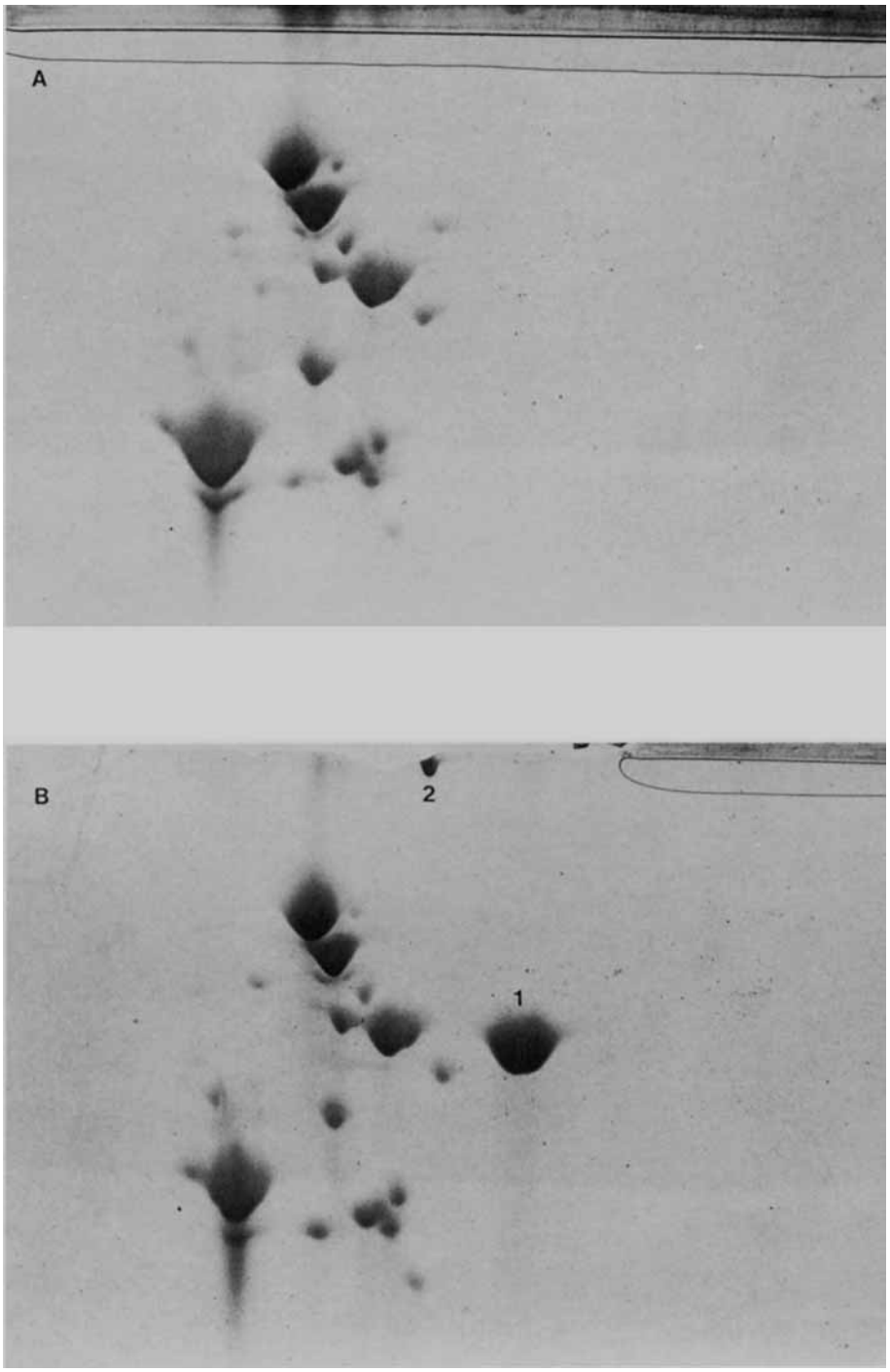

Fig. 4. Two-dimensional polyacrylamide gel electrophoresis of shock fluids of strain G 810/3 (A) and G 810/3, 3-46 (B). Spot No. 1 is monomeric GLPT, and No. 2 is tetrameric GLPT. Conditions as described in the legend to Fig. 1.

2. Properties of GLPT

A. Quaternary structure. GLPT was purified from the osmotic shock fluid (Boos et al., 1977). The rational of the purification procedure was based on the properties of GLPT during 2-dimensional polyacrylamide gel electrophoresis, i.e., a molecular weight of 40,000 and a rather acidic isoelectric point. Therefore, GLPT was purified by Sephadex G-100 chromatography followed by preparative isoelectric focusing (pI 4.4).

In the absence of an adequate enzymatic test, 2-dimensional polyacrylamide electrophoresis was used to monitor the purification. Two problems were encountered: 1) the presence of a proteolytic activity that splits GLPT into a peptide of 35,000 daltons, and 2) the fact that GLPT in the native form exhibits quaternary structure and is composed of

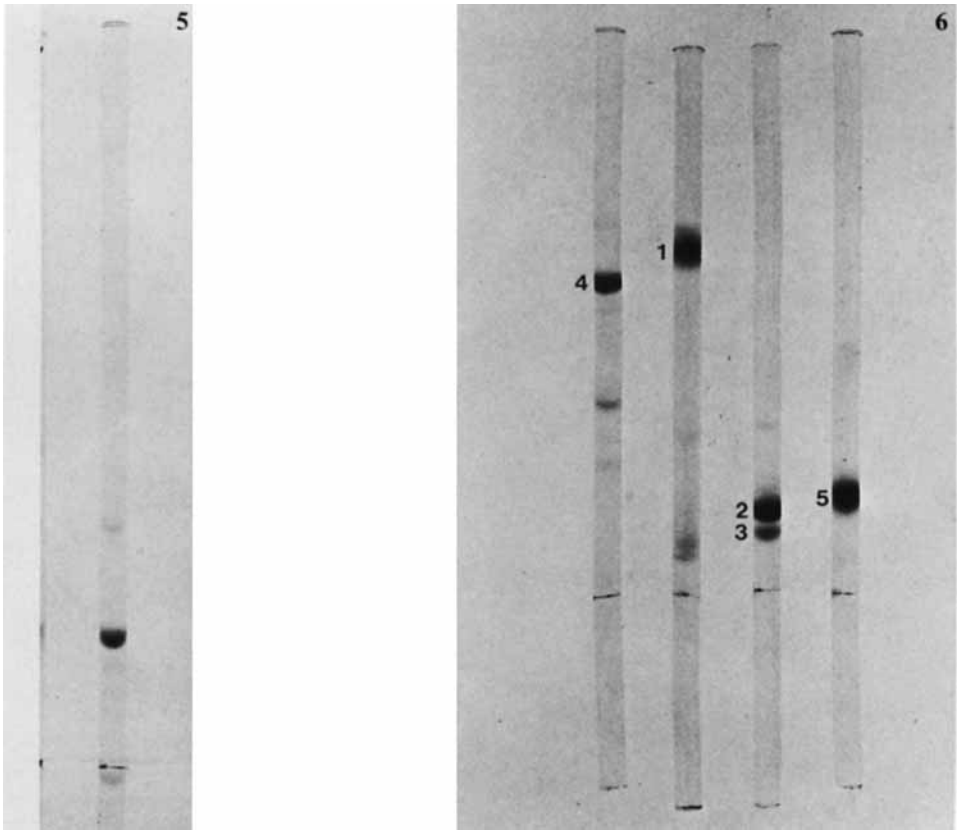

Fig. 5. Polyacrylamide gel electrophoresis in SDS of a purified preparation of GLPT. The sample was boiled for 10 min in 1% SDS, 1% mercaptoethanol. About 15μg protein were applied. The gels contained 5% acrylamide. The ink mark indicates the migration of bromphenol blue. Electrophoresis was performed at 8 mA per tube for 5 h. The direction of migration is from top to bottom. Taken from Boos et al. (1977) with the permission of authors and publisher.

Fig. 6. Polyacrylamide gel electrophoresis in SDS of purified preparations of GLPT with and without boiling in SDS. The samples were boiled for 10 min in 1% SDS, 1% mercaptoethanol. About 20 μg protein were applied. 1) Protein GLPT, unboiled sample; 2, 3) protein GLPT, boiled sample; 4) E. coli β-galactosidase; 5) chicken serum albumin. Experimental conditions as described in the legend to Fig. 5. Taken from Boos et al. (1977) with the permission of authors and publisher.

4 identical subunits of approximately 40,000 molecular weight. The purest preparation analyzed by the usual SDS electrophoresis is shown in Fig. 5. In this case the sample had been boiled in 1% SDS prior to electrophoresis. In contrast, merely incubating the sample in SDS without boiling prior to electrophoresis reveals that GLPT occurs as a complex with molecular weight of 160,000. Figure 6 demonstrates this phenomenon and compares the 2 different species of GLPT with marker proteins of molecular weight 135,000 (E. coli β-galactosidase) and 45,000 (chicken serum albumin). In contrast to the GLPT preparation seen in Fig. 5, the preparation used here was stored at 4°C for several days. As a consequence proteolytic degradation of GLPT occurred which was revealed by the additional presence of a polypeptide of 35,000 molecular weight after boiling of the 160,000 molecular weight complex. Since this proteolytic digestion occurred in the 160,000 molecular weight complex and does not result in an apparent reduction in the molecular weight of the complex its quaternary structure remains intact despite the proteolytic degradation.

Dissociation of the GLPT complex can easily be followed by SDS-polyacrylamide gel electrophoresis in crude shock fluid since the tetrameric form of GLPT is by far the slowest moving band in this technique. This is demonstrated in Fig. 7. Dissociation of the

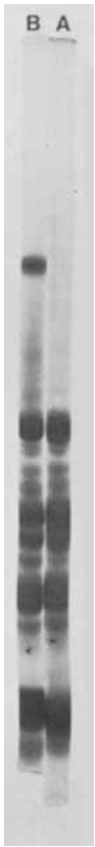

Fig. 7. Polyacrylamide gel electrophoresis in SDS of crude shock fluid of strain G 810/3, 3-46. A) Sample boiled in SDS for 10 min prior to electrophoresis, B) untreated sample.

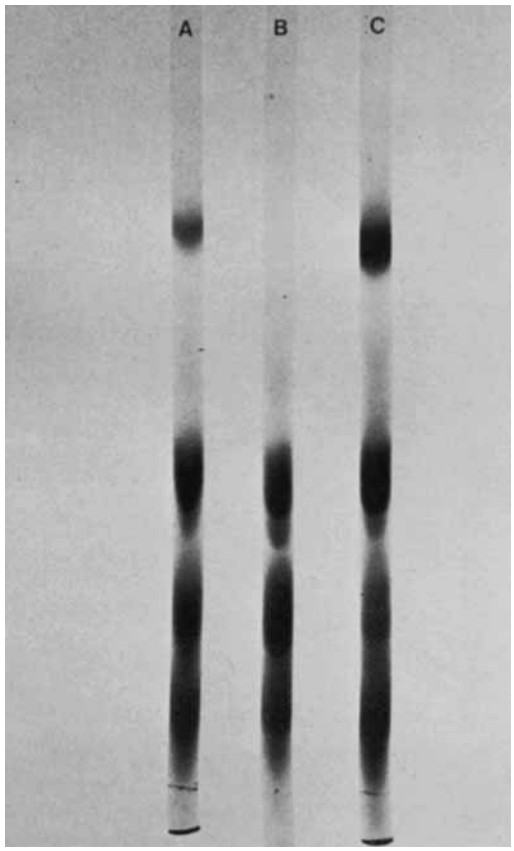

Fig. 8. Polyacrylamide gel electrophoresis in SDS of crude shock fluids of strain G 810/3, 3-46. A) Treated for 30 min with 8 M urea, followed by dialysis against 100 mM Tris-HCl, pH 7.0; B) treated for 30 min in 8 M urea; C) untreated sample. About 150 μg protein were applied on the gels. Experimental conditions as described in the legend to Fig. 5.

complex by 8 M urea or boiling SDS is reversible. Dialysis overnight of the treated sample against 10 mM Tris-HCl buffer, pH 7, results in reformation of the complex. This is shown in Fig. 8.

Cross-linking of the GLPT complex by a series of diimidates of varying chain length and subsequent analysis of the cross-linked products after boiling in SDS reveals that only a species of twice the molecular weight of the monomer can be linked together (not shown).

This dimeric species cannot be seen in experiments where the GLPT complex is exposed for 30 min to 1% SDS at different temperatures. While dissociation is nearly complete at temperatures above 40°C, at temperatures below 40°C only the monomeric and tetrameric but not the dimeric form can be seen. Due to the analysis of shock proteins by 2-dimensional electrophoresis involving initial separation of the proteins in 8 M urea, we at first had not realized the tetrameric nature of GLPT. However, reexamination of these gels, particularly of shock proteins derived from a *glpT* plasmid carrying strain, revealed the existence of the tetrameric form of GLPT on 2-dimensional gels. This can easily be seen on Figs. 2 and 4 (spots No. 2) To avoid artifactual appearance of multiple spots

caused by long exposure to urea (carbamylation by cyanate) we routinely added urea to the protein sample only shortly before electrophoresis in the first dimension. Dissociation of GLPT under these conditions is apparently slow and is not completed during the time of the electrophoretic run. Dissociation is completely stopped after the electrophoresis in the second dimension is begun and the protein freed from urea. This is obvious since no trailing of spot No. 2 (GLPT complex) occurs in the second dimension. This again demonstrates the surprising resistance of the GLPT complex against SDS at room temperature.

B. Tests for possible enzymatic activity. From the genetic correlation concerning the map positions of the different enzymes contained in the *glp* regulon, the identity of GLPT with glycerolkinase or with either of the catabolic flavin-linked and membrane-bound glycerolphosphate dehydrogenases was excluded. Tests performed with GLPT to detect any enzymatic activity of glycerolphosphatase or glycerolkinase activity were negative. Similarly, equilibrium dialysis using [^{14}C]-labeled G3P as ligand did not reveal any binding activity of GLPT towards G3P under a variety of different conditions of ionic strength and metal ions. The experimental setup was such that a dissociation constant of 10^{-4} M or less could be detected. For these experiments purified GLPT had to be used since crude preparations contain a phosphatase activity that hydrolyzed G3P to glycerol and inorganic phosphate.

3. Cell-Division-Dependent Transport Activity for G3P; Precursor or Positioning Mechanism for GLPT

Studies by Ohki (1972) demonstrated that transport activity of G3P in E. coli was subject to a cell-cycle-dependent regulation. Similar phenomena have been found with the transport activity of the β-methylgalactoside system (Shen and Boos, 1973). In the latter system the corresponding periplasmic substrate recognition site, the galactose-binding protein, was similarly affected in its synthesis. Synthesis of material cross-reacting with anti-galactose-binding protein antibodies occurred during cell division but not during cell elongation. Similar observations were made in BUG-6, a mutant which is temperature sensitive for cell division (Reeve et al., 1970). This strain synthesized galactose-binding protein only at the permissive temperature (Shen and Boos, 1973). Therefore, it was of interest to determine if GLPT synthesis would exhibit the same phenomena. Figure 9 shows an experiment where several parameters were measured during synchronized cell growth: 1) optical density, 2) cell number, 3) transport of G3P, 4) rate of total protein synthesis (TCA-precipitable material), 5) rate of GLPT synthesis (EDTA-lysozyme-sucrose extractable [^{14}C]-labeled material that comigrates with tetrameric GLPT on SDS gels).

This experiment clearly showed the cell-division-dependent alteration of transport activity for G3P but did not reveal a corresponding alteration in the rate of synthesis for the tetrameric form of GLPT as recovered by the lysozyme-EDTA-sucrose method.

This would mean that it cannot be the periodically altering synthesis of GLPT that evokes the cell-division-dependent alteration in G3P transport activity. However, it might be caused by a cell-division-dependent processing of a GLPT precursor, or its cell-division-dependent positioning into a functional state in the cell envelope. Experiments with BUG-6, the temperature-sensitive cell division mutant, are relevant in this respect. This strain grows and divides normally at 35°C. When shifted to 42°C, division stops immediately and long filaments form. The mutation does not affect DNA replication but prevents septum formation (Reeve et al., 1970).

The BUG-6 mutant when grown overnight at 42°C on glycerol does not exhibit any transport activity for G3P, while cultures grown at 35°C transport normally. Analysis of

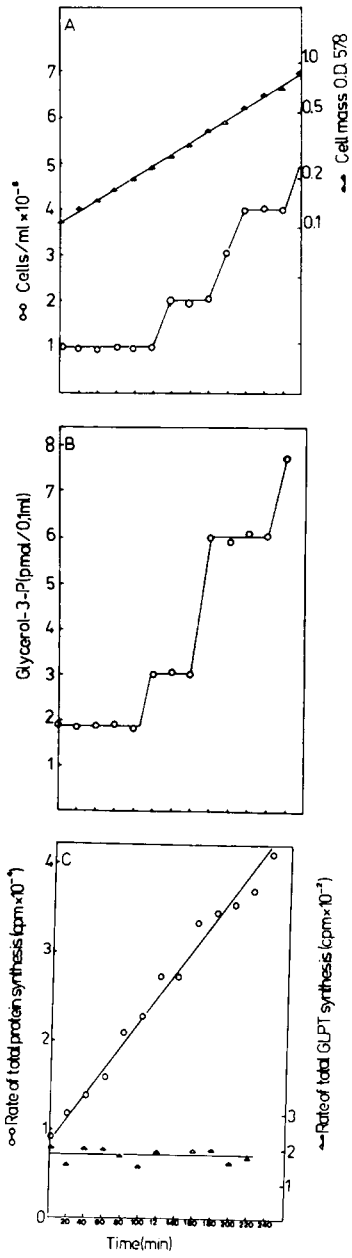

Fig. 9. Net transport activity and GLPT synthesis in synchronized cells of strain 72. A) Growth was followed by measuring the optical density at 578 nm. Cell number was measured by plating for single cell colonies on nutrient broth agar plates. B) Transport activity (initial rate of uptake) is given in pmol of [^{14}C]-G3P taken up per 30 sec per 0.1 ml of growth culture. C) Pulse labeling of the GLPT was done for 10 min with [U-^{14}C]-leucine, [U-^{14}C]-valine and [U-^{14}C]-alanine followed by a 10 min chase with excess of unlabeled amino acids. Incorporation of radioactivity is given in cpm per 0.1 ml EDTA-lysozyme-20% sucrose extractable [^{14}C]-labeled material that comigrates with tetrameric GLPT on SDS gels. Rate of total protein synthesis is followed by the incorporation of radioactivity in TCA precipitable material.

the periplasmic shock proteins released from both cultures revealed the following: Both preparations contained large amounts of material that in the immunodiffusion technique cross-reacts with anti-GLPT antibodies. However, only the preparation grown at the permissive temperature contained GLPT as judged by 2-dimensional gel electrophoresis. Therefore, the cross-reactivity to GLPT must be caused by a GLPT precursor.

When a culture of BUG-6 that had been growing for 50 min at the nonpermissive temperature is shifted to the permissive temperature under simultaneous addition of chloramphenicol, increase of transport activity is observed (Fig. 10). Since cell division can be initiated under these conditions (Reeve and Clark, 1972) the cell-cycle-dependent processing of GLPT is very likely responsible for the observed alteration in transport activity in normal strains. It also suggests that the primary temperature-sensitive defect in BUG-6 is contained in a proteolytic enzyme.

At the present time it is not clear why BUG-6 releases the precursor of GLPT upon osmotic shock. It also is not clear at what location in the cell envelope the processing occurs. From the shock releasability of the precursor one would think that processing occurs in the periplasm. However, from the cell-division-dependent correlation of this phenomenon, processing on or within the cytoplasmic membrane is a more likely picture. In this respect it is relevant to refer to the shock releasability of the elongation factor Tu, which is pictured to be connected to the inner site of the cytoplasmic membrane (Jacobson and Rosenbusch, 1976).

4. Transport of G3P in Membrane Vesicles, the G3P Carrier

Previous experiments by other workers have shown that G3P can be transported actively in membrane vesicles. This is surprising in view of our finding of the periplasmic nature of GLPT, namely, that it is one of the gene products of the *glpT* operon. If GLPT is an essential component of the G3P transport system, in analogy to many periplasmic substrate-binding proteins (Boos, 1974), membrane vesicles should be unable to transport G3P. However, this is not the case. Figure 11 shows the transport activity of 3 membrane vesicle preparations: from a fully induced wild-type strain, from a constitutive strain, and from a mutant that does not transport G3P and lacks GLPT. As can be seen the transport activity observed in whole cells is analogous to the transport activity measured in their isolated membrane vesicles. Therefore, either transport in membrane vesicles is mediated by residual GLPT in these membranes, or GLPT is only essential for transport of G3P in whole cells but not in membrane vesicles. Two-dimensional polyacrylamide gel electrophoresis of the proteins contained in these membrane vesicles did not reveal any residual amounts of GLPT (not shown). Therefore, it is clear that GLPT is not essential for the energy dependent active transport of G3P in membrane vesicles, and its only function must reside in overcoming a possible diffusion barrier for G3P in the outer envelope structure of E. coli.

As a consequence it is clear that membrane vesicles must contain a protein, different from GLPT, that is coded for by the *glpT* operon and that is responsible for the active transport of G3P, the "G3P carrier." The above-mentioned electrophoretic analysis of the proteins contained in membrane vesicles did not show any difference in the protein pattern between a wild-type strain and one that should not contain the G3P carrier. However, the availability of strains that contained *glpT* carrying plasmids (and thus must produce more of the G3P carrier) together with the use of an extraction procedure specific for proteins of the inner (cytoplasmic) membrane (Schnaitman, 1971), made possible the identification of a protein that is a very likely candidate for the G3P carrier protein. Figure 12 shows

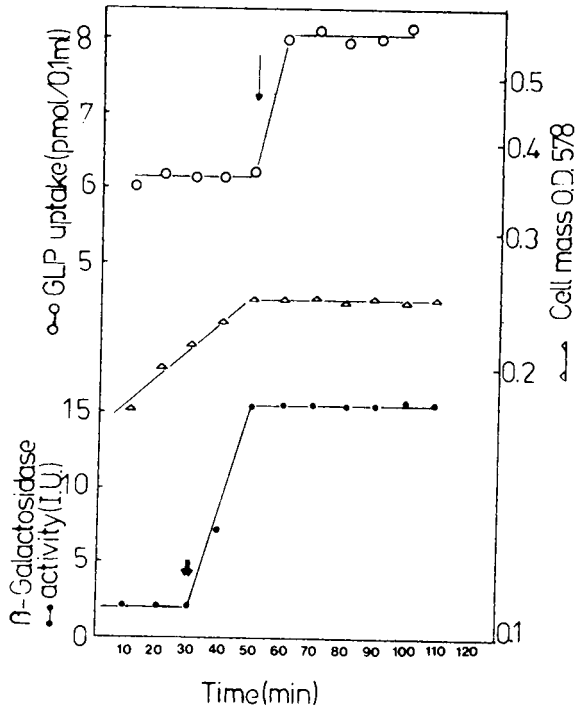

Fig. 10. Effect of chloramphenicol on G3P transport activity of E. coli BUG-6. The culture was grown for 50 min at 42°C. At the time indicated by the arrow the temperature of the culture was shifted to 35°C with simultaneous addition of chloramphenicol (150 μg/ml final concentration). Growth and transport assay as described in the legend to Fig. 9. β-galactosidase activity, induced at the time indicated by the double arrow, was assayed as described by Ullman et al. (1968).

the electrophoretic analysis of proteins contained in the cytoplasmic membrane of a transport-negative strain (it does not contain GLPT) in the presence and absence of the *glpT* carrying plasmid. As can be seen, there are minor quantitative differences in the amounts of several proteins. However, the plasmid-carrying strains contain one spot (x) that is absent in the *glpT* strain without plasmid.Therefore, this protein must be coded for by one of the plasmid genes. The purification of this protein and its test for binding activity toward G3P will be necessary to establish it as the as yet unknown G3P carrier.

5. Some Periplasmic Proteins Are Accessible From the Outside Medium

Since GLPT does not participate in the energy dependent translocation step of G3P in the cytoplasmic membrane it is likely to be connected at least functionally to the outer envelope structure of the Gram negative E. coli cell.

If by some structural arrangement in the outer membrane GLPT would facilitate the diffusion of G3P it is not unlikely that at least some portion of its polypeptide chain should be extended to or be in contact with the external medium. To test this possibility lactoperoxidase mediated iodination with [^{125}I] was performed with whole cells of E. coli. Figure 13 shows the 2-dimensional polyacrylamide gel electrophoresis pattern of the periplasmic shock proteins released after the iodination procedure. After staining, all spots were cut from the gel, dissolved in H_2O_2-ammonia, and counted in the liquid

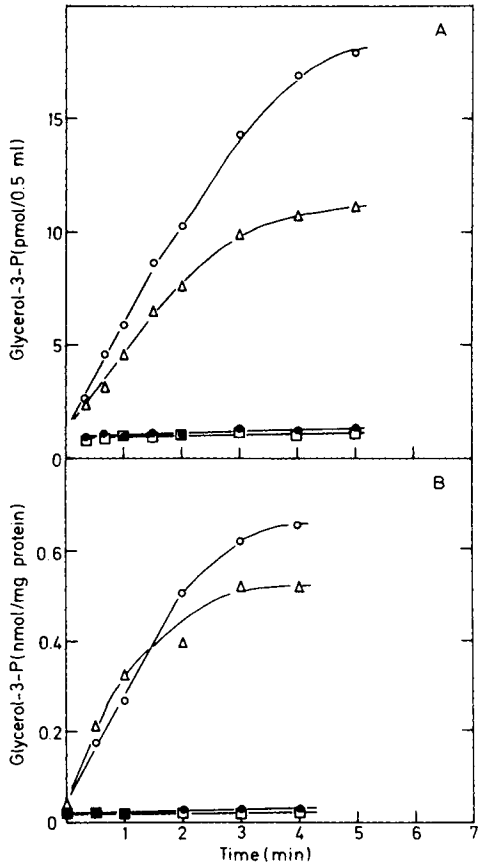

Fig. 11. Transport of G3P in whole cells and membrane vesicles of wild-type and *glpT* mutants of E. coli. A) Uptake in whole cells. The bacterial cultures were resuspended in 100 mM Tris-HCl, pH 7.0, to an absorbance of 1.0 at 578 nm. [^{14}C]-G3P was added at an initial concentration of 80 nM. The data are expressed in terms of amounts G3P taken up per 0.5 ml samples. All operations were done at room temperature. B) Uptake in membrane vesicles. The uptake mixture contained per filtered aliquot 70 μg membrane protein; initial [^{14}C]-G3P concentration was 0.8 μM, and D-lactate was added at a final concentration of 20 mM. ○) Constitutive strain 72; △) inducible strain LA 3400; ●) *glpT* mutant LA 3404; □) *glpT* mutant LA 3404. All strains were grown with glycerol as carbon source. Taken from Boos et al. (1977) with the permission of authors and publisher.

scintillation counter. The counts that were obtained are indicated on Fig. 13. Surprisingly, many periplasmic proteins were labeled. Although GLPT is among the labeled proteins, its radioactivity is only 15% of the most heavily labeled proteins. The fact that not all proteins were labeled throughout demonstrates that this method is specific for surface located proteins and indicates such a position also for GLPT.

DISCUSSION

In this paper we discussed the different aspects of GLPT, a protein that is closely related to the transport system of G3P in E. coli. All available evidence points to the conclusion that GLPT is coded for by one of the genes contained in the *glpT* operon.

Mutants that lack GLPT map within the *glpT* region. Suppressible nonsense mutations in *glpT* or Mu insertions in *glpT* affect the amount of GLPT produced. Finally, plasmids that contain the *glpT* region produce large amounts of GLPT in a strain that otherwise lacks this protein. At the present time we do not have a *glpT* mutant carrying a nonsense mutation in GLPT that we could identify as such on polyacrylamide gels. Therefore, we cannot yet exclude the possibility that GLPT production might be dependent on a hypothetical positive regulator that is coded for by one of the *glpT* genes. If such is the case, the structural gene for GLPT could be located anywhere on the E. coli chromosome.

In comparison to other periplasmic proteins GLPT has some unusual properties. It consists of 4 identical subunits of 40,000 molecular weight. This GLPT complex is stable in 1% SDS at room temperature. Treatment with 8 M urea or with SDS at temperatures above 40°C dissociates the complex. This denaturation is reversible by simple dialysis against Tris or phosphate buffers at pH 7.0.

Until now the relationship of GLPT to G3P transport has been indirect, based on the genetic correlation that has been discussed. Clearly, GLPT is not similar to the numerous periplasmic substrate-binding proteins that have been identified as the recognition sites for active transport systems. In contrast to these proteins GLPT does not exhibit binding activity towards its supposed substrate nor are membrane vesicles devoid of the corresponding transport activity. Therefore, the only remaining function for GLPT in G3P transport is to act as a facilitator for the diffusion of G3P through the outer membrane of E. coli. The observation that GLPT can be iodinated by lactoperoxidase in intact cells indicates that GLPT could have access to the outer surface of the cell.

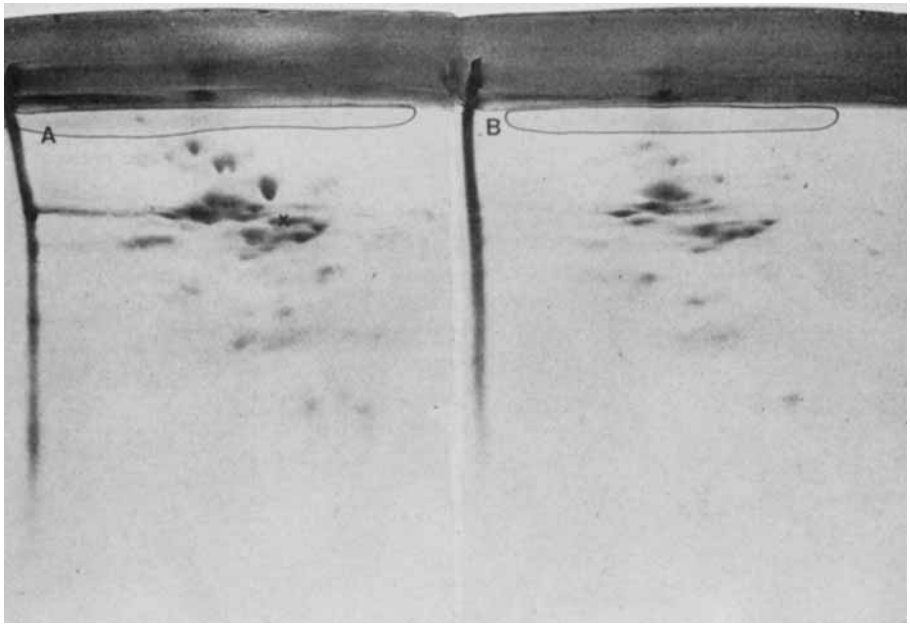

Fig. 12. Two-dimensional polyacrylamide gel electrophoresis of proteins contained in the cytoplasmic membrane of the *glpT* strain G 810/3 (B), and its *glpT*$^+$ plasmid carrying derivative (A). Both strains were grown with glycerol as carbon source. The proteins were extracted from the cell envelope by Triton X-100 in the presence of $MgCl_2$. Two hundred micrograms protein were applied on the gels.

No information as to the in vivo location or structure of GLPT is as yet available. In particular there is no evidence for the pore-like structure which one might propose for a protein that facilitates the diffusion of a substrate through an otherwise hydrophobic membrane. However, one cannot fail to note the highly acidic nature of GLPT. Using isoelectric focusing, a pI of 4.4 was found for GLPT. This is reflected in the fast electrophoretic mobility of GLPT in gels containing urea. Possibly the abundance of negative charges in GLPT at neutral pH is connected to its function in G3P transport.

As with other periplasmic proteins very little is known concerning either its biosynthesis or its translocation through the cytoplasmic membrane. The electrophoretic

Fig. 13. Two-dimensional polyacrylamide gel electrophoresis of crude shock fluid of strain 72 after lactoperoxidase catalyzed iodination with [^{125}I] of intact cells. The Coomassie Blue stained spots were cut out from the gel, dissolved in H_2O_2-ammonia, and counted. The numbers depicted on the spots represent their counts per minute. Background activity is 15–20 cpm.

analysis of the shock proteins from a mutant that is temperature sensitive for cell division demonstrates, at least for this strain, the existence of a GLPT precursor. This precursor undergoes proteolytic processing before it attains its final form. The picture is even further complicated by the observation that newly formed capacity for G3P transport appears to be correlated to events occurring only at certain times during the cell cycle. If this phenomenon is related to GLPT at all, it must be the cycle-dependent processing of this protein, but not its synthesis, that is causing the effect. At present it is not clear where in the cell envelope this processing takes place or whether it is related to the translocation of GLPT through the cytoplasmic membrane.

Since GLPT is not necessary for active transport in membrane vesicles the *glpT* operon must code for at least one more protein, the G3P carrier. Indeed, the electrophoretic analysis of proteins contained in the cytoplasmic membrane did reveal a polypeptide that might be the as yet unknown G3P carrier. This indication is based on the

observation that this protein is produced in a *glpT* strain only after the introduction of the *glpT* carrying plasmid. The biochemical characterization and identification of this protein as the G3P carrier will have to await its certainly very challenging purification.

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