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Cell-Free Synthesis of Proteins Related to *sn*-Glycerol-3-Phosphate Transport in *Escherichia coli*

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An *Escherichia coli* periplasmic protein (GlpT) related to *sn*-glycerol-3-phosphate transport was synthesized in a cell-free system directed by hybrid plasmic ColE1-*glpT* DNA. The in vitro product cross-reacted with antisera against the purified protein. The ColE1-*glpT* DNA-directed cell-free system was induced by *sn*-glycerol-3-phosphate and phosphonomycin and was dependent on cyclic AMP. The in vitro-synthesized protein showed the characteristics of a multimeric protein, as did the purified periplasmic protein. The main proportion of the newly synthesized product had a higher molecular weight than the mature protein found in the periplasm of cells and showed a more positive charge in two-dimensional gel electrophoresis. Thus, a proportion of this protein is presumed to be synthesized in vitro as a precursor. The cell-free system yielded a second protein that is likely to be also coded for by the *glpT* operon. This protein had a molecular weight of approximately 33,000 in sodium dodecyl sulfate-acrylamide gel electrophoresis and behaved like an intrinsic membrane protein.

A group of proteins exists in gram-negative bacteria that is assumed to be located between the inner and outer membrane in the periplasmic space. The main evidence for their location in the periplasm is the observation that these proteins are released from the cell by the cold osmotic shock procedure of Neu and Heppel (14).

Among these proteins are the numerous substrate-binding proteins that do not show any enzymatic activity but bind small molecules like sugars and amino acids. These binding proteins are substrate recognition sites for active transport systems, and some of them function in addition as chemoreceptors (3).

Periplasmic proteins have to be synthesized by ribosomes in the cytoplasm, by processes that are very poorly understood, prior to translocation into the periplasm.

In eucaryotic systems, excreted proteins are synthesized on ribosomes associated with membranes through which the proteins are to pass (2). Studies in cell-free systems lead to the hypothesis that the structural genes for the excreted proteins contain a signal sequence that codes for hydrophobic amino acids. This short hydrophobic peptide mediates the attachment of the ribosome-peptide complex to the inner membrane. The proteins are then elongated across the inner membrane, and the signal peptide is removed by a proteolytic enzyme (2).

The procaryotic periplasmic protein alkaline phosphatase has recently been shown to be synthesized in a cell-free system as part of a precursor with a higher molecular weight than is known for the mature enzyme. This precursor protein can be processed to approximately the correct size by an outer membrane fraction (8). In addition, in vivo synthesis of this enzyme through the cytoplasmic membrane of spheroplasts via membrane-bound ribosomes has directly been demonstrated (19). It has recently also been shown that another extracellular protein, the murein lipoprotein, is synthesized in vitro as part of a precursor (9). These results suggest some similarities between the excretion of proteins in procaryotes and eucaryotes.

A hybrid ColE1 plasmid carrying the *Escherichia coli glpT* operon has been constructed (6). In vivo experiments using this hybrid ColE1-*glpT* plasmid provided evidence that this plasmid could code for proteins involved in glycerol-3-phosphate transport as well as synthesis of the GlpT protein in a mutant *glpT* strain (1).

Here we report the cell-free synthesis, directed by hybrid ColE1-*glpT* DNA, of the periplasmic GlpT protein and of a second protein which sediments with the membrane fraction of the cell-free extract.

MATERIALS AND METHODS

Bacterial strains. *E. coli* 74/3 is a derivative of CSH74 made *glpT* by selecting for phosphonomycin resistance (21); the *glpT* marker is cotransducible with *nalA* (18). *E. coli* JA200 (*thr leu* Δ*trpE5 recA* F$^+$) containing hybrid ColE1 plasmid pLC3-46 or 14-12 (6)

was kindly supplied by L. Clarke and J. Carbon. The number, for example 3-46, identifies the plasmid in the colony bank and signifies that this particular plasmid is located on plate 3, patch 46 in the collection. *E. coli* F165λ(λd*gal*) was used to prepare λd*gal* DNA (13).

Conditions for cell-free protein synthesis. DNA from hybrid plasmid ColE1-*glpT* was prepared as described by Clarke and Carbon (6). The procedures for preparation of λd*gal* DNA have been described by Wetekam et al. (22). The conditions for cell-free protein synthesis and preparation of the 30,000 × *g* cell-free extract (S30) were according to Zubay et al. (23) with the modifications described by Schumacher and Ehring (17). Synthesis was performed in a 0.2-ml reaction mixture for 1 h at 37°C, followed by 1 h of incubation at the same temperature in the presence of deoxyribonuclease (25 μg/ml, final concentration). The reaction mixture contained 44 mM tris(hydroxymethyl)aminomethane (Tris)-acetate (pH 8.2), 1.4 mM dithiothreitol, 55 mM potassium acetate, 27 mM ammonium acetate, 2.2 mM ATP, 0.55 mM each GTP, UTP, and CTP; 0.22 mM each of 20 amino acids, 21 mM phosphoenolpyruvate, 7.4 mM $CaCl_2$, 0.5 mM cyclic AMP (cAMP), 10 mM $MgCl_2$, 1 mM glycerol, and 2 U of glycerokinase. The optimum amount of cell-free extract (S30) added to the reaction mixture was determined for each preparation as measured by incorporation of [^{14}C]leucine into acid-precipitable material in the DNA-directed cell-free system.

ColE1-*glpT* DNA was added to the cell-free system at a concentration ranging from 20 to 40 μg/ml. For measuring induction of the *glpT* operon by *sn*-glycerol-3-phosphate or phosphonomycin, a DNA concentration of 1.5 and 5 μg/ml, respectively, was used. Blank values were obtained by incubating a reaction mixture without DNA. For labeling the newly synthesized proteins and subsequent identification in polyacrylamide gels, methionine was omitted from the reaction mixture and 25 μCi of [^{35}S]methionine (100 Ci/mmol; Amersham) per ml was added.

When [^{14}C]leucine was used to label the in vitro-synthesized proteins, nonlabeled leucine was added at a concentration of 0.055 mM instead of the normally used 0.22 mM, and [^{14}C]leucine was added at a concentration of 0.25 μCi/ml (270 mCi/mmol; Amersham).

Separation of the in vitro reaction mixture into particulate and soluble fractions. Cell-free protein synthesis was performed in a 0.4-ml reaction mixture. After 1 h of incubation, 25 μg of deoxyribonuclease and 25 μg of ribonuclease were added to the reaction mixture, and incubation was continued for another 10 min. The in vitro sample was then made 20 mM in ethylenediaminetetraacetate and centrifuged for 45 min at 40,000 rpm in a Beckman Ty 65 rotor. The sediment was resuspended in 0.4 ml of 0.025 M Tris-hydrochloride (pH 8.3). The particulate fraction and the supernatant were then dialyzed against 0.025 M Tris-hydrochloride (pH 8.3) overnight.

Synthesis and identification of *sn*-glycerol-3-phosphate. *sn*-Glycerol-3-phosphate was synthesized under conditions of cell-free protein synthesis. The reaction mixture contained, in addition, glycerol at a concentration of 10 mM, [γ-^{32}P]ATP (Amersham) at a radioactive concentration of 5 μCi/ml, and 2 U of glycerokinase (Sigma). After 30 min of incubation at 37°C, 0.2 ml of ice-cold 10% trichloroacetic acid was added; the samples were centrifuged for 10 min at 5,000 × *g*, and 3 μl of the supernatant was used for chromatographic separation on a polyethyleneiminocellulose precoated plate (Merck). A 1.5-μCi sample of *sn*-[*U*-^{14}C]-glycerol-3-phosphate (New England Nuclear) was used as the standard. Chromatography was performed with a solvent containing 75 ml of 2-propanol, 25 ml of water, 0.25 ml of ammonia (25%), and 5 g of trichloroacetic acid. After drying, the plate was subjected to autoradiography with Kodak Definix medical film for 15 h.

SDS-polyacrylamide gels. After 2 h of incubation of the cell-free reaction mixtures, samples were dialyzed against 0.025 M Tris-hydrochloride (pH 8.3) overnight. Samples were made 1 mM with respect to dithiothreitol and 1% with respect to sodium dodecyl sulfate (SDS) and boiled at 100°C for 10 min or kept at room temperature as indicated in the figure legends. Samples of 50 μl were applied to a 12.5% SDS-polyacrylamide slab gel similar to that described by Studier (20). The electrophoresis buffer was according to Laemmli (11) and contained 0.025 M Tris, 0.19 M glycine (pH 8.3), and 0.1% SDS. Electrophoresis was done for 16 h at 75 V at a final amperage of 5 mA. Purified unlabeled GlpT protein was used as a standard. Gels were stained with Coomassie brilliant blue for 2 h and destained overnight. After drying, gels were subjected to autoradiography with Kodak XS-1 film or Kodak Definix medical film for 3 days.

Two-dimensional polyacrylamide gel electrophoresis. Conditions for two-dimensional polyacrylamide gel electrophoresis were as described (10, 18).

Immunodiffusion assay. Rabbit antiserum against purified GlpT was placed in the center well of an immunodiffusion plate (Immuno-plate, Hyland Div., Travenol Laboratories). An 8-μl sample of the 200-μl cell-free reaction mixture was placed in the peripheral well with enough nonradioactive carrier protein to give strong precipitation lines with the antiserum. After incubation for 2 h at 37°C, the immunodiffusion plates were incubated in 200 ml of 2% sodium chloride overnight, stained with Coomassie brilliant blue, and destained overnight. The plates were dried and subjected to autoradiography for 1 week using Kodak XS-1 film.

Measurement of [^{14}C]leucine incorporation into acid-precipitable material. Cell-free protein synthesis was stopped by the addition of 1 ml of cold water to the 0.2-ml in vitro reaction mixture. Proteins were precipitated by the addition of 1 ml of ice-cold 10% trichloroacetic acid. Samples were kept on ice for 20 min and then heated for 20 min at 95°C to hydrolyze charged tRNA (15). The precipitates were collected on glass fiber filters [Whatman GF (B)] and washed with 4 volumes of 5 ml each of cold 2% trichloroacetic acid. Filters were dried, and radioactivity was measured in a toluene-based scintillation mixture in a scintillation counter (Intertechnique).

Measurement of radioactivity in gel bands from polyacrylamide gels. Bands from gels corresponding to darkened areas on films were cut out. The gel pieces were digested in scintillation vials using 0.5 ml of hydrogen peroxide (30%) and 0.05 ml of ammonia

(25%). The vials were incubated at 42°C overnight. Radioactivity was measured in Bray solution (5).

RESULTS

***glpT*-DNA-directed cell-free protein synthesis.** Figure 1 shows the time-dependent incorporation of [^{14}C]leucine into acid-precipitable material in the hybrid ColE1-*glpT* plasmid DNA-directed cell-free synthesis. A linear increase of newly synthesized proteins was observed from 5 to 30 min, and the maximum accumulation of trichloroacetic acid-precipitable radioactive proteins was observed at 50 min. Control samples that contained no DNA showed an incorporation of 7.5% of the maximum incorporation in the presence of DNA. The maximum accumulation of [^{14}C]leucine in trichloroacetic acid-precipitable material represented 40% of the added labeled leucine to the in vitro reaction mixture.

Regulation of gene expression of the *glpT* operon. The hybrid ColE1-*glpT* plasmid codes for the transport system for *sn*-glycerol-3-phosphate transport including the periplasmic GlpT protein (1). The expression of GlpT protein in vivo is induced by glycerol as a carbon source, while the true inducer is *sn*-glycerol-3-phosphate that is produced internally from glycerol (for review, see 12). Our experiments using glycerol-3-phosphate as an inducer for the *glpT* operon in the cell-free system failed to show induction, probably because of the presence of phosphatases in the S30 extract. Addition of glycerol-3-phosphate to the in vitro system caused inhibition of the overall protein synthesis (data not shown). Instead of glycerol-3-phosphate, glycerol and glycerokinase were added to the cell-free system. These two components, together with ATP, generated *sn*-glycerol-3-phosphate under conditions of cell-free protein synthesis (Fig. 2). As can be seen from Table 1, the protein-synthesizing capacity of hybrid ColE1-*glpT* DNA was stimulated about twofold by glycerol plus glycerokinase. This low rate of induction is not surprising, since *sn*-glycerol-3-phosphate transport in *E. coli* is stimulated only three- to fourfold when cells are grown on glycerol instead of succinate.

Induction of the *glpT* operon was also measured using phosphonomycin as the inducer. Mutants resistant to this drug were found to be defective in the uptake of *sn*-glycerol-3-phosphate or glucose-6-phosphate (21). Since this antibiotic is recognized by the *sn*-glycerol-3-phosphate transport system, it may as well be recognized by the *glpR*-encoded repressor molecule. Table 2 shows that phosphonomycin in fact can be used to stimulate *glpT* DNA-directed gene expression in vitro. In this experiment, again, protein synthesis was induced by a factor of two. In a control experiment, it was shown that λd*gal* DNA-dependent protein synthesis is unaffected by phosphonomycin.

From in vivo data it is known that the *glp*

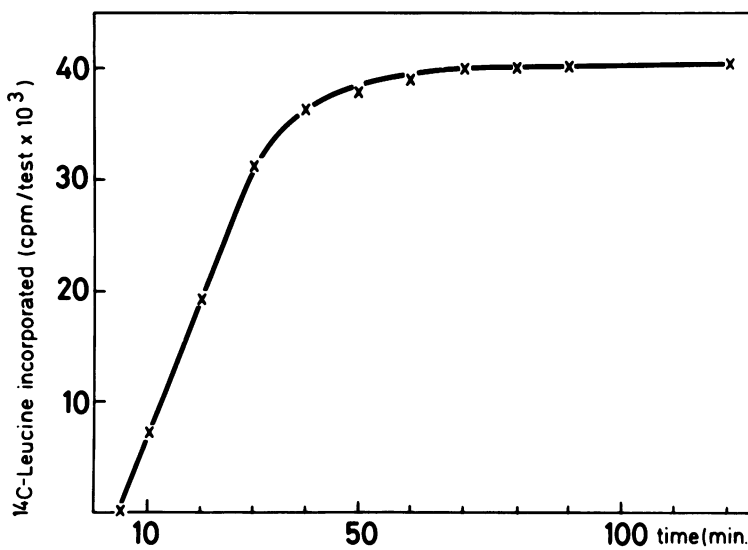


FIG. 1. *Incorporation of [^{14}C]leucine in glpT DNA-directed cell-free system. Conditions for cell-free protein synthesis and measurement of incorporation of [^{14}C]leucine into acid-precipitable material were as described in the text. A 40-μg/ml concentration of hybrid ColE1-glpT DNA was used to direct cell-free protein synthesis. The values have been corrected by subtracting a blank value of 3,110 cpm.*

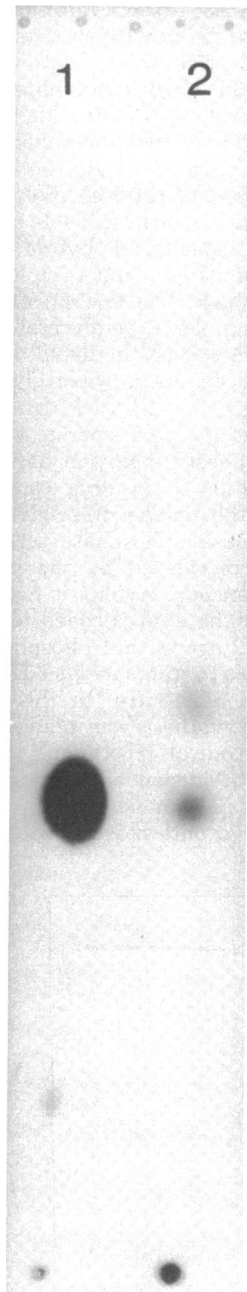


FIG. 2. *Synthesis of sn-glycerol-3-phosphate in the cell-free system. The reaction mixture contained all components of the cell-free system including ColE1-glpT DNA (20 μg/ml). Conditions for synthesis of sn-glycerol-3-phosphate and autoradiography were as described in the text. Lane 1, sn-Glycerol-3-phosphate used as standard; lane 2, sn-glycerol-3-phosphate synthesized in the presence of glycerol, glycerokinase, and [γ-^{32}P]ATP in the cell-free system.*

TABLE 1. *Induction of total protein synthesis directed by glycerol and glycerokinase*[a]

Conditions for cell-free synthesis	[^{14}C]Leucine incorporated (cpm/test)
Control	1,169
+Glycerol	1,449
+Glycerokinase	1,085
+Glycerol + glycerokinase	2,733

[a] Hybrid ColE1-*glpT* DNA was used to direct cell-free protein synthesis. The incubation mixture designated as control contained neither glycerol nor glycerokinase. Where indicated, 1 mM glycerol and/or 2 U of glycerokinase were added. The values have been corrected by substracting a blank value of 2,110 counts per minute (cpm). Data are the averages of three independent experiments.

TABLE 2. *Induction of total protein synthesis by phosphonomycin*[a]

Cell-free protein synthesis directed by:	[^{14}C]Leucine incorporated (cpm/test)
ColE1-*glpT* DNA	
−Phosphonomycin	6,100
+Phosphonomycin	12,700
λd*gal* DNA	
−Phosphonomycin	6,100
+Phosphonomycin	6,400

[a] Conditions for cell-free protein synthesis were as described in the text. Where indicated, phosphonomycin (1 mM, final concentration) was added to the reaction mixture. The values have been corrected by subtracting a blank value of 1,500 counts per minute (cpm). (A different cell-free extract was used for this experiment. It was more active in directing cell-free protein synthesis than that used for the experiment shown in Table 1.)

regulon is catabolite repressible (for review, see 12). If the hybrid plasmid pLC3-46 carries the regulator region of the *glpT* operon, one should expect *glpT* DNA-directed protein synthesis to be stimulated by cAMP in the presence of the catabolite gene activator protein.

Figure 3 shows that the overall protein synthesis directed by hybrid ColE1-*glpT* DNA was stimulated about threefold by the addition of cAMP. When radioactively labeled in vitro-synthesized proteins were separated in an SDS-polyacrylamide gel, the band corresponding to the GlpT protein was cut out, and radioactivity was measured, a sixfold stimulation by cAMP, from 7,800 to 47,000 cpm, was observed.

Immunodiffusion assay. A sample of [^{35}S]-methionine-labeled in vitro-synthesized proteins was tested in an immunodiffusion assay using antisera against purified GlpT protein (Fig. 4).

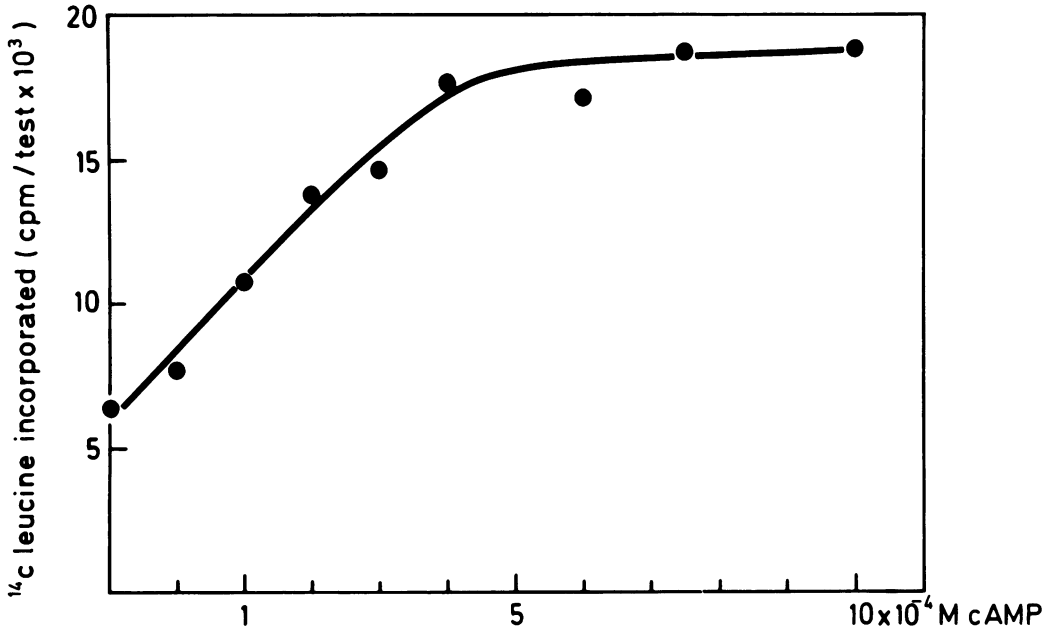


FIG. 3. *cAMP-dependent incorporation of [^{14}C]leucine into acid-precipitable material. A 20-μg/ml concentration of hybrid ColE1-glpT DNA was used to direct cell-free protein synthesis. cAMP was added to the reaction mixture at concentrations as indicated in the abscissa. All values have been corrected by subtracting a blank value of 1,990 cpm.*

A strong precipitin line was observed when an aliquot of the complete reaction mixture was used for the immunodiffusion assay. When cAMP was omitted from the reaction mixture, only a very faint precipitin line was observed.

Characterization of in vitro-synthesized products by gel electrophoresis. As was published previously, the GlpT protein is a multimeric protein. The subunit form has a molecular weight of 40,000 (18). Treatment of the GlpT protein with SDS at room temperature or up to 40°C does not completely dissociate the multimeric form of the protein, whereas boiling at 100°C in 1% SDS does (4). These characteristics were taken as further criteria for identification of the newly synthesized GlpT protein. Figure 5B shows a stained slab gel in which a sample of the in vitro reaction mixture and purified unlabeled GlpT protein were analyzed. Figure 5A shows the corresponding autoradiograph of the unboiled (1) and the boiled sample (2). As can be seen, the sample kept at room temperature in 1% SDS showed two bands of the higher-molecular-weight complex (a, b). These bands disappeared after boiling. The molecular weight of the minor band (d) corresponds to the nonradioactive GlpT subunit (slot 4), while the major band (c) has a molecular weight higher by 1,000 to 2,000. Since both the complex and the subunit form of the protein exhibited the same characteristics upon treatment with SDS as the purified nonradioactive GlpT protein, we conclude that GlpT is synthesized in vitro as precursor, of which only a small proportion is modified to the mature protein. It should be noted that the migration of GlpT protein on SDS-polyacrylamide gels after treatment in SDS at different temperatures is very characteristic for this protein. As can be seen in Fig. 5B, slot 1 and 2, and in contrast to GlpT (slots 3 and 4), no changes in the band patterns of the proteins of the S30 extract were observed under these conditions.

Characterization of in vitro-synthesized gene products in two-dimensional gel electrophoresis. To get more information about the nature of the proteins synthesized in the *glpT*-DNA-directed system, proteins were analyzed using two-dimensional polyacrylamide gel electrophoresis.

Here, proteins were separated first according to electrical charge in the presence of urea and then according to size in the presence of SDS. Proteins of the same molecular weight were found on the same horizontal line, and proteins of the same electrical charge appeared on the same vertical line. From comparison of Fig. 6A and 6B, the position of the mature unlabeled

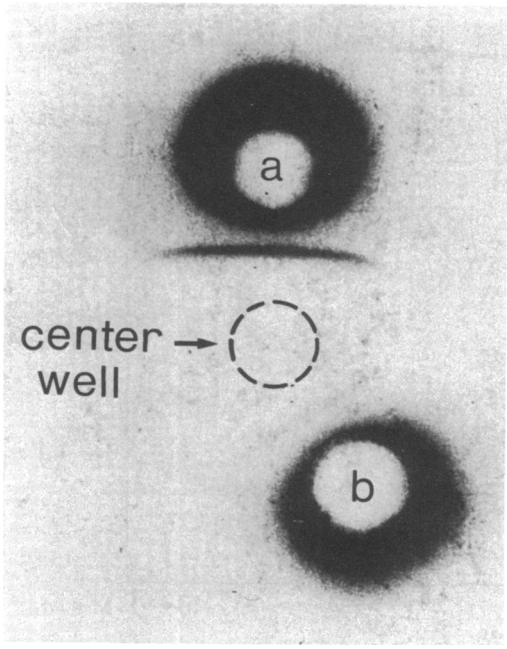


FIG. 4. *Autoradiograph of an Ouchterlony immunodiffusion plate showing cross-reacting material of in vitro-synthesized proteins. The center well contained 8 μl of antiserum against purified GlpT. The outer wells contained the following: (a) 8 μl of a complete reaction mixture; (b) 8 μl of a reaction mixture from which cAMP was omitted. Enough unlabeled GlpT protein was added to both wells to give strong precipitin lines.*

GlpT protein in the two-dimensional gel system could be identified. (Homogeneous GlpT protein exhibited double spots on two-dimensional polyacrylamide gels, as if heterogeneous in electrical charge. The reason for this charge heterogeneity is not clear.) The autoradiograph of the in vitro-synthesized products separated in the same gel system shows one protein exactly at the position of the mature GlpT (indicated by the arrow) and a second one, the putative precursor, that is more positively charged than the mature GlpT (Fig. 7).

The differences in molecular weight between the GlpT precursor and the mature GlpT, as shown in the SDS-polyacrylamide gel (Fig. 5A), could not be detected in the two-dimensional gel system. This was not surprising, since separation according to size was done only over a short range in the two-dimensional gel system.

Identification of a presumptive membrane protein coded for by the *glpT* operon. Another protein (Fig. 8, band e) of a molecular weight of about 33,000 was synthesized in the *glpT*-DNA-directed system only when cAMP was present in the reaction mixture. Several proteins that were synthesized in the absence of cAMP disappeared after addition of cAMP. This is probably due to an increase of initiation of transcription at the cAMP-dependent *glpT* operon which may reduce the expression of cAMP-independent operons under the limiting conditions of a cell-free system. The protein (band e) is assumed to be coded for by the *glpT* operon, since experiments using ColE1 DNA instead of hybrid ColE1-*glpT* DNA to direct cell-free protein synthesis showed no stimulation of gene expression by cAMP (data not shown). As a first step to characterize this protein, the in vitro reaction mixture was separated into particulate and soluble fractions by centrifugation. The protein sedimented almost quantitatively with the particulate fraction (Fig. 9, lane 2) and could therefore be a membrane protein. As a control, the proteins encoded by the galactose operon were synthesized in a λd*gal* DNA-directed cell-free system. After separation of the cell-free system into particulate and soluble fractions, the λd*gal* DNA-encoded proteins were found almost exclusively in the supernatant (Fig. 9, lane 3). Therefore, the presence of this *glpT* DNA-directed protein in the particulate fraction is characteristic for its properties and not an artifact of in vitro-synthesized proteins.

DISCUSSION

Hybrid plasmid ColE1-*glpT* DNA was used as a template to direct cell-free synthesis of the *glpT* operon-encoded proteins. Overall *glpT* DNA-directed protein synthesis was induced by *sn*-glycerol-3-phosphate or by phosphonomycin and stimulated by cAMP. These data demonstrate that the hybrid ColE1-*glpT* plasmid pLC3-46 carries the regulator region of the *glpT* operon. On the other hand, these results show that phosphonomycin, which is recognized by the *sn*-glycerol-3-phosphate transport system (21), can also act as inducer of the *glpT* operon.

One of the proteins synthesized under cell-free conditions is the GlpT protein, which has been characterized as a periplasmic protein (18). The in vitro-synthesized GlpT protein has been identified by immunodiffusion assay and through its characteristic behavior in SDS-gel electrophoresis and two-dimensional gel electrophoresis. A large proportion of the in vitro-synthesized GlpT protein is presumably the precursor of the mature protein. This conclusion is based on the observation that two bands of the higher-molecular-weight complex disappeared upon boiling in SDS. These proteins reappeared in two dif-

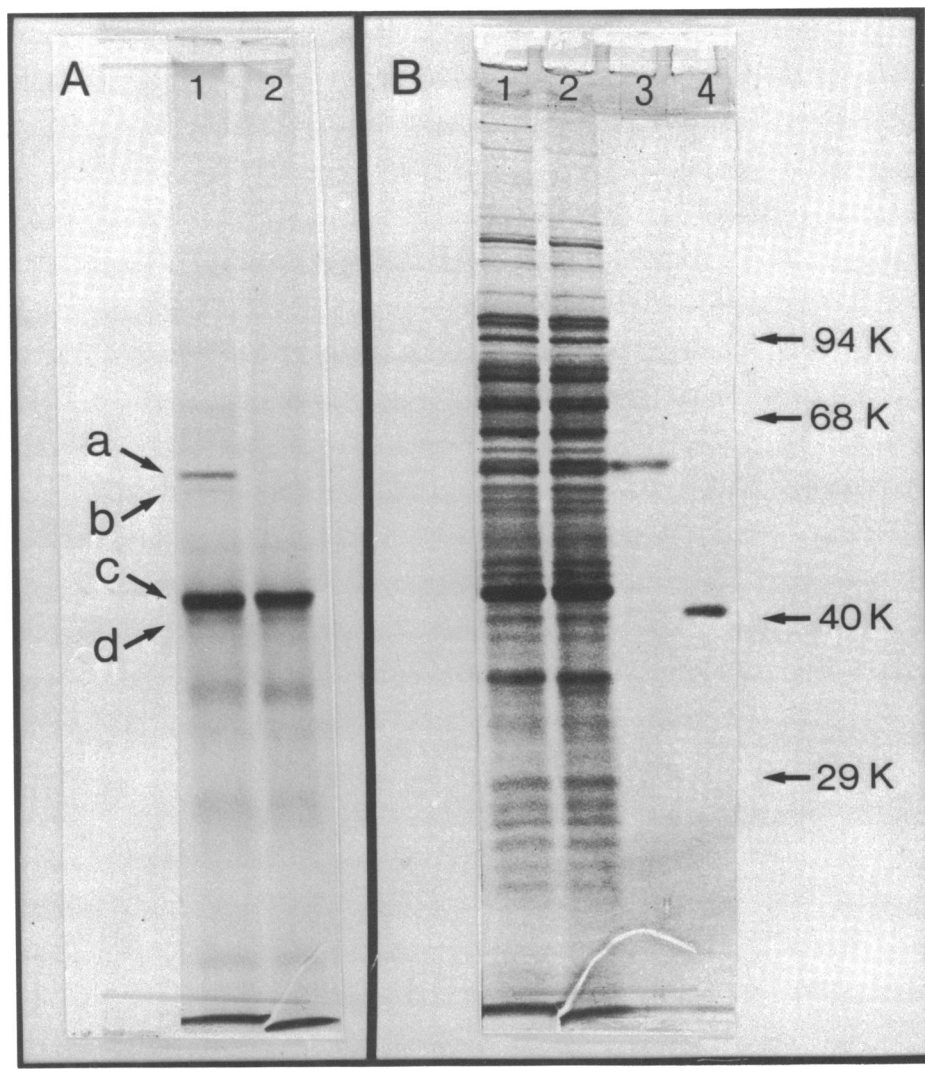
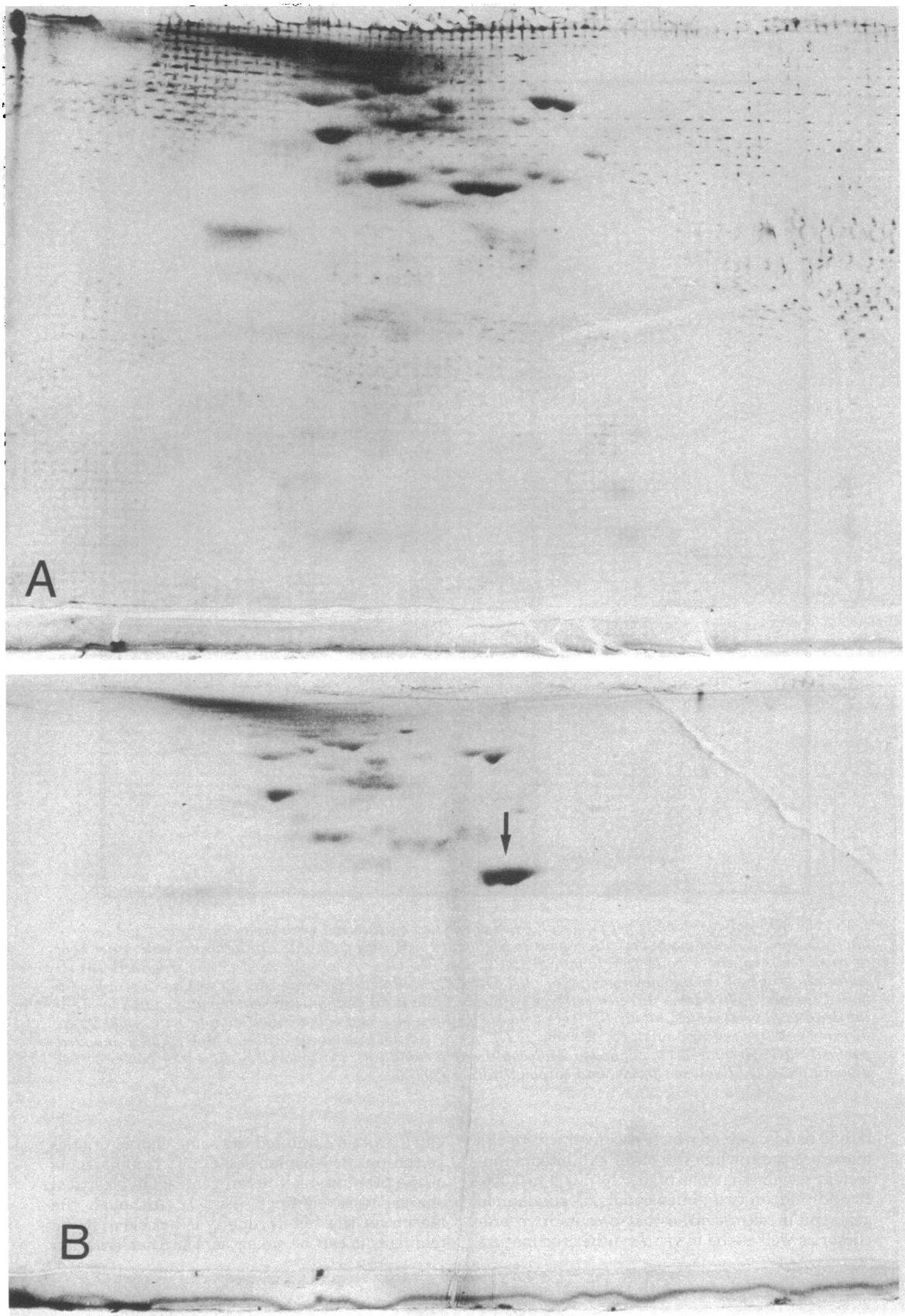


FIG. 5. *SDS-polyacrylamide gel electrophoresis of in vitro-synthesized gene products. Prior to gel electrophoresis, samples were made 1% with respect to SDS and 1 mM with respect to dithiothreitol and either kept at room temperature or boiled for 10 min at 100°C. Purified GlpT (5 μg) was used as the standard. (A) (1) Autoradiograph of an unboiled sample of glpT-DNA-directed cell-free synthesis; (2) autoradiograph of a boiled sample of glpT-DNA-directed cell-free synthesis. Lane 1: (a) and (b) represent the aggregated forms of the denatured proteins (c) and (d). (B) (1) Coomassie brilliant blue-stained unboiled sample of the glpT-DNA-directed cell-free reaction mixture; (2) same as (1), but boiled; (3) Coomassie brilliant blue-stained unboiled purified GlpT; (4) same as (3), but boiled. Molecular weight standards: phosphorylase A (94,000), bovine serum albumin (68,000), aldolase (40,000), and carboanhydrase (29,000).*

ferent bands, one of which was identical to the mature protein while the other exhibited a molecular weight that was higher by 1,000 to 2,000. In addition, on two-dimensional polyacrylamide gels, the in vitro-synthesized presumptive precursor as well as the in vitro-synthesized mature GlpT protein exhibited the same characteristics as the purified nonlabeled GlpT. Double spots of the proteins, as if heterogeneous in electrical charge, were obtained (Fig. 7). Although the reason for this heterogeneity is not clear at the moment, it can be taken as a further criterion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B

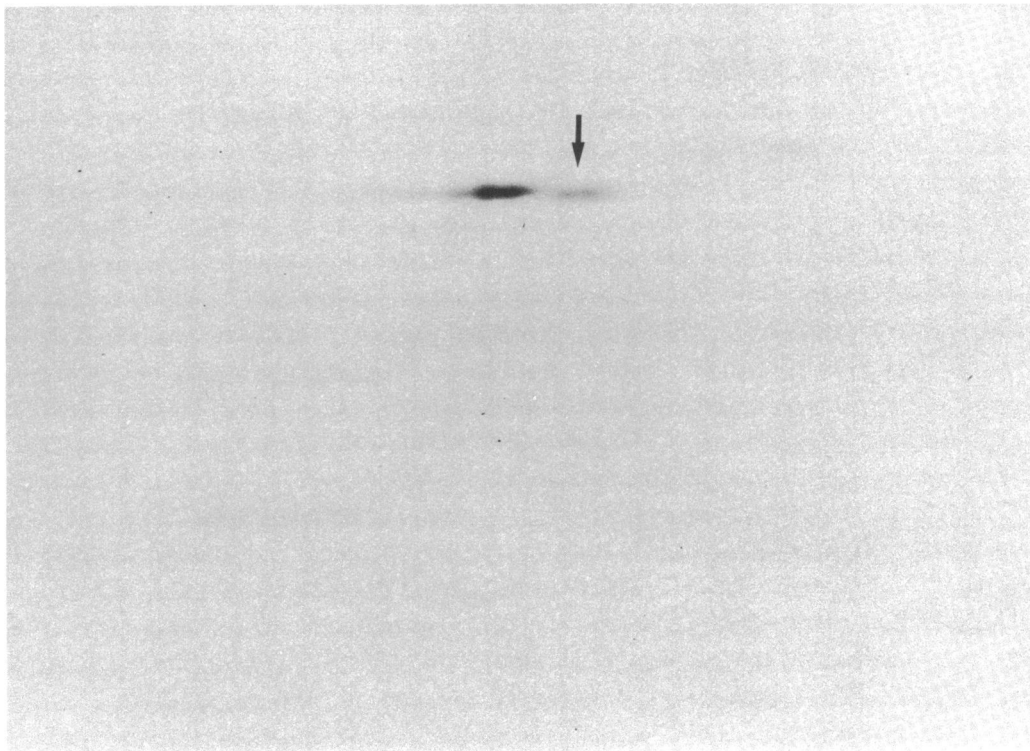

FIG. 7. *Two-dimensional gel electrophoresis of in vitro-synthesized gene products. A 50-μl sample of a glpT-DNA-directed cell-free reaction mixture was used for separation in two-dimensional gel electrophoresis. Gels were stained, dried, and subjected to autoradiography. The arrow indicates the position of the purified unlabeled GlpT protein.*

for the GlpT protein-like nature of the precursor molecule.

Recently, two other procaryotic proteins, periplasmic alkaline phosphatase (8) and the murein lipoprotein (9), have been shown to be synthesized in vitro as precursor as well. These findings are consistent with the signal hypothesis originally postulated for the excretion of eucaryotic proteins (2). According to this hypothesis, excreted proteins are coded for by structural genes that enclose the message for a hydrophobic peptide at the amino-terminal end. The hydrophobic peptide mediates the attachment of the ribosome peptide complex to the membrane. Proteins are then elongated across the membrane, followed by the cleavage of the signal peptide by a proteolytic enzyme.

Synthesis of murein lipoprotein (7) and in vitro protein synthesis by membrane-bound polysomes has been found to exhibit a higher resistance against puromycin than the synthesis of cytoplasmic proteins (16). This was partly explained by the association at the ribosome peptide complex with the membrane (7). Our experiments using the *glpT* DNA-directed cell-free system and, as control, λd*gal* DNA-directed cell-free protein synthesis showed no difference in the inhibition by puromycin (data not shown).

Since the 30,000 × *g* supernatant (S30), which was used as a source for transcription and translation, still contained membranes, the cell-free reaction mixtures could be separated into a particulate and a soluble fraction. The in vitro-synthesized protein with an apparent molecular weight of 33,000 sedimented almost quantitatively with the particulate fraction (Fig. 9). This protein might therefore be an intrinsic membrane protein. In addition, this protein could not

FIG. 6. *Two-dimensional gel electrophoresis of the in vitro reaction mixture. Gels were stained with Coomassie brilliant blue. The following was applied to the gel: (A) 50 μl of the glpT-DNA-directed cell-free reaction mixture or (B) 50 μl of the glpT-DNA-directed cell-free reaction mixture together with 50 μl of purified nonradioactive GlpT (12 μg). The arrow indicates the position of GlpT protein.*

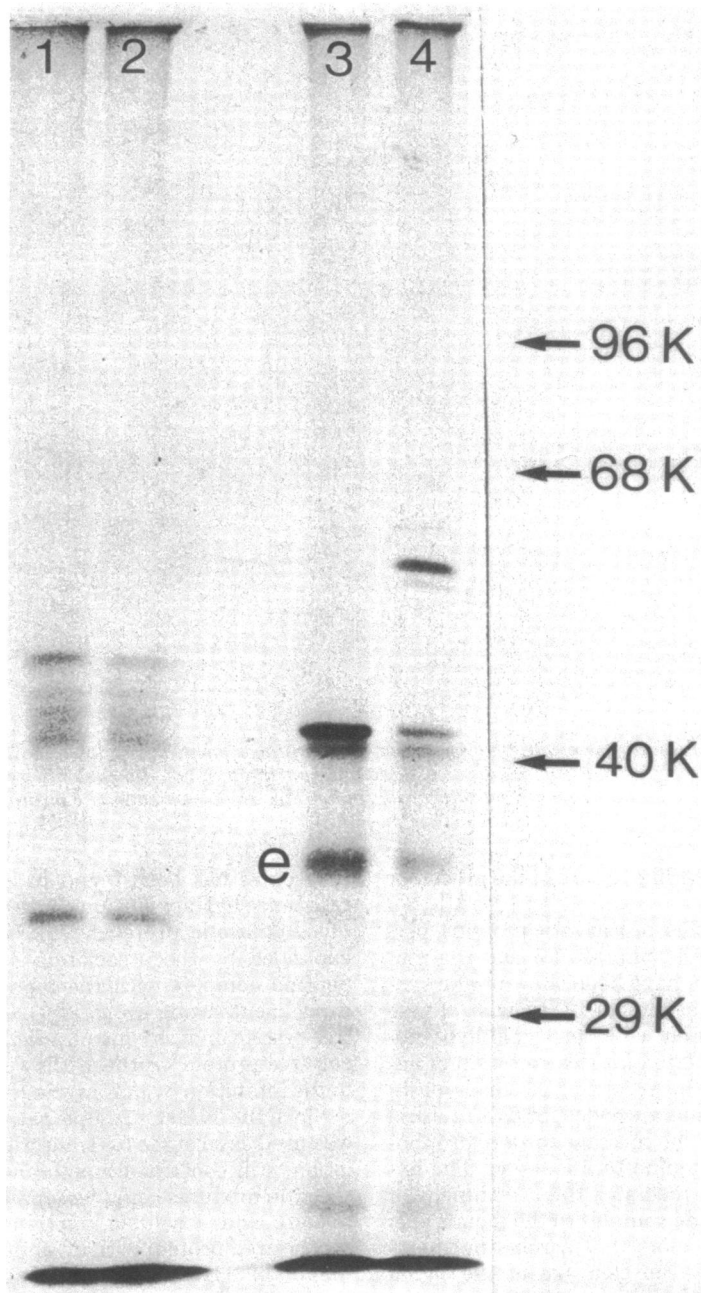

FIG. 8. *Synthesis of glpT-DNA-encoded gene products in dependence on cAMP. A 50-μl portion of a glpT-DNA-directed cell-free reaction mixture was used for separation in SDS-gel electrophoresis. Samples were taken from a cell-free reaction mixture from which cAMP was omitted, made 1% with respect to SDS and 1 mM with respect to dithiothreitol, and either boiled for 10 min at 100°C (1) or kept at room temperature (2). The same treatment was done with a sample of a glpT-DNA-directed cell-free reaction mixture which contained cAMP. The sample was either boiled (3) or kept at room temperature (4).*

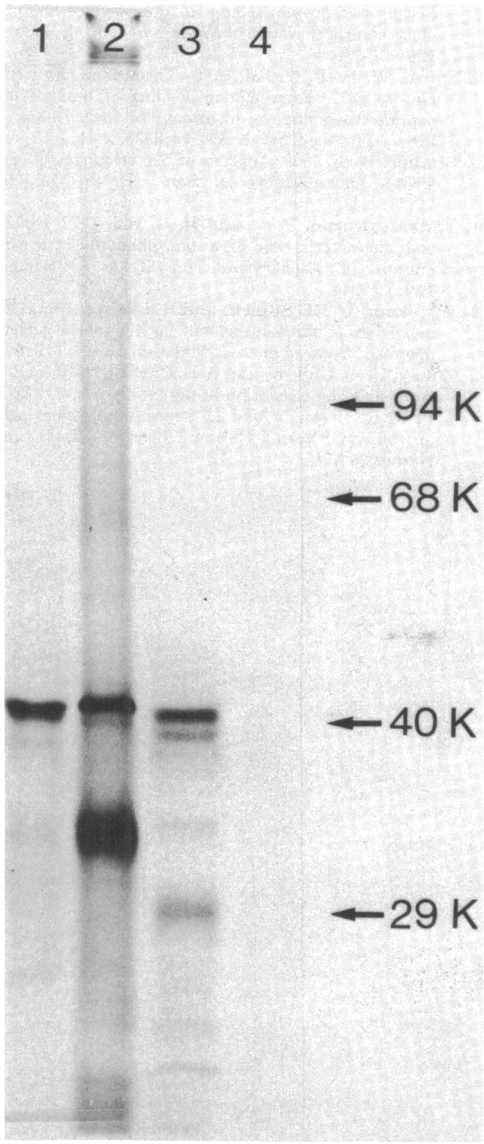

FIG. 9. *Separation of in vitro-synthesized gene products into particulate and soluble fractions. Hybrid ColE1-glpT DNA or λdgal DNA was used to direct cell-free protein synthesis. Proteins were separated into soluble and particulate fractions and subjected to gel electrophoresis as described in the text. Autoradiograph of glpT-DNA-directed cell-free reaction mixture separated into (1) soluble and (2) particulate fraction; autoradiograph of λdgal DNA-directed cell-free reaction mixture separated into (3) soluble and (4) particulate fraction.*

be detected by two-dimensional gel electrophoresis. In this system, membrane proteins do not enter the urea gel (first dimension) without prior dissolution in chaotropic agents (10). These findings do not conclusively show that this protein is a membrane protein, but the fact that in vitro-synthesized proteins in a λd*gal*-directed cell-free system are almost exclusively found in the supernatant shows that sedimentation in the particulate is characteristic for this protein. Further experiments are required to clarify the properties of this protein, in particular its possible identity with the membrane-bound carrier of *sn*-glycerol-3-phosphate transport.

Besides this presumed membrane protein, a fraction of the protein that showed the characteristics of the periplasmic GlpT protein sedimented with the particulate fraction.

At the moment we cannot distinguish between the possibilities (i) that a proportion of GlpT protein is membrane bound or (ii) that GlpT is synthesized across membranes and a fraction of it remains attached to the membrane.

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