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Reconstitution of Binding Protein Dependent Active Transport of Glutamine in Spheroplasts of *Escherichia coli*†

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ABSTRACT: In order to directly prove that the periplasmic glutamine binding protein is an essential component of the osmotic shock sensitive active transport system for glutamine in *Escherichia coli*, we demonstrated the reconstitution of binding protein dependent glutamine transport in spheroplasts of that organism. It was shown by arsenate inhibition that the reconstituted transport system was energy dependent, and the use of azaserine, an inhibitor of glutamine-utilizing enzymes, indicated that the restoration of transport by binding protein did not require the metabolizing of the transport substrate. Furthermore, the binding protein dependent transport of glutamine was shown to require at least one other macromolecular component, presumably membrane bound, which was absent in a strain containing a deletion of the genes coding for the glutamine transport system but was present in a strain carrying a mutation only in the structural gene for the glutamine binding protein.

Despite a wealth of genetic evidence that the periplasmic binding proteins of *Escherichia coli* and *Salmonella typhimurium* are associated with particular active transport systems [for examples, see Ames & Lever (1970), Boos (1972), Rahmanian et al. (1973), and Weiner & Heppel (1971)], direct demonstrations of this fact have been scarce. Shortly after the discovery of many of the binding proteins, a number of studies appeared reporting the restoration of various transport systems in osmotically shocked cells by the addition of concentrated shock fluid or purified binding proteins (Wilson & Holden, 1969; Anraku, 1968; Medveczky & Rosenberg, 1970; Nishimune & Hayashi, 1971). However, the extent of the binding protein stimulated transport in shocked cells was, in most cases, not substantial, and proper control experiments were not demonstrated. In addition, some of these results could not be reproduced by other investigators (Rosen, 1973) or even by the same laboratory (Gerdes et al., 1977). In order to avoid these ambiguities, we examined the possibility of restoring shock-sensitive glutamine transport in spheroplasts of *E. coli*. In this paper we discuss the reconstitution of binding protein dependent active transport of glutamine in spheroplasts and show that the reconstituted transport system is energy dependent and does not require the metabolizing of the transport substrate. Evidence is also presented that glutamine transport requires at least one other macromolecular component in addition to the glutamine binding protein.

Materials and Methods

Materials. Radioactive glutamine was from New England Nuclear; radioactive proline was from Schwarz/Mann. Cellulose acetate filters, used in transport assays, were obtained from Millipore; nitrocellulose filters, used in glutamine binding protein assays, were from Schleicher & Schuell. Sucrose was Schwarz/Mann special enzyme grade. Azaserine (*O*-diazoacetyl-L-serine) was obtained from Calbiochem.

Bacterial Strains and Growth Media. The three *E. coli* K-12 strains used in this study were PSM2 (F⁻ *thi metC glnPo*), PSM223 (F⁻ *thi metC ΔnadA glnP*), and PSM116 (F⁻ *thi metC glnPo glnP*), which will be described in detail elsewhere (unpublished results). PSM2 contains a regulatory mutation, *glnPo*, that causes overproduction of the glutamine transport system. PSM223, derived from PSM2, contains a deletion extending entirely through the genes coding for the glutamine transport system. PSM116, also derived from PSM2, has a point mutation in the structural gene for the glutamine binding protein.

Minimal salts medium E (Vogel & Bonner, 1956) containing 0.5% succinate (sodium salt) was used for growth of cells. Methionine was added to 0.4 mM, thiamine to 40 μM, nicotinamide to 30 μM, and biotin to 20 nM.

Purification of the Glutamine Binding Protein. Glutamine binding protein was purified as described previously (Willis & Seegmiller, 1976), except that the final column (SP-Sephadex) was eluted with 4 column volumes of a linear gradient of 0.05-0.20 M NaCl in 10 mM Tris[1]-acetate, pH

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5.0, followed by 4 column volumes of 0.20 M NaCl in 10 mM Tris–acetate, pH 5.0. The purified glutamine binding protein was judged homogeneous by $NaDodSO_4$–polyacrylamide gel electrophoresis by a modification of the procedure of Laemmli (Laemmli, 1970); no contaminating bands were seen when as much as 20 μg of protein was loaded onto the gel. The purified binding protein had an apparent molecular weight of 25 000 and a specific activity of 16.6 nmol of glutamine bound/mg of protein, determined by using a nitrocellulose filter binding assay (Lever, 1972). The preparation contained no detectable amount of the glutamate–aspartate binding protein (Willis & Furlong, 1975), which copurifies with glutamine binding protein through a number of steps, as measured by the binding of glutamate. Glutamine binding protein was stored in liquid nitrogen and used within 5 months of its isolation.

Preparation of Spheroplasts. Lysozyme–EDTA spheroplasts were prepared essentially by the method of Kaback with modifications due to the sensitivity of *E. coli* K-12 to lysozyme (Kaback, 1971). Cells grown on minimal succinate medium at 37 °C were harvested at a density of 3.5×10^8 cells/mL by centrifugation at 12000*g* for 10 min and were washed twice with cold (8–10 °C) 10 mM Tris-HCl, pH 8.0, and 0.6 g of cells (wet weight) was resuspended in 50 mL of 30 mM Tris-HCl, pH 8.0, and 20% sucrose. K-EDTA, pH 7.0, and lysozyme were added to 10 mM and 50 μg/mL, respectively, and the suspension was stirred at room temperature (23 °C) for 30 min. $MgSO_4$ was then added to 15 mM, DNase and RNase were added, each to 100 μg/mL, and stirring was continued for an additional 10 min. Spheroplasts were collected by centrifugation, washed once with 50 mM Tris-HCl, pH 7.0, containing 20% sucrose, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, and 1 mM $MgSO_4$ (buffer A), and finally resuspended to 20 mL in buffer A containing 100 μg/mL chloramphenicol. This suspension had a protein concentration in the range of 2.0–2.5 mg/mL. Spheroplasts were stored on ice, and transport assays were performed immediately.

Preparation of Vesicles. Bacterial membrane vesicles were prepared exactly as described by Kaback (Kaback, 1971), except that the concentration of lysozyme used was reduced to 50 μg/mL. When small molecular weight compounds were to be incorporated into the vesicles, these were included in the hypotonic buffer into which spheroplasts were burst and also in all buffers used in the subsequent washes and resuspensions. Vesicles were stored on ice, and transport was assayed on the same day as preparation.

Transport Assays. For assay of transport by spheroplasts, a volume of spheroplast suspension was mixed with an equal volume of either buffer A or binding protein which had been dialyzed against buffer A overnight at 0 °C. Aliquots (100 μL) of this suspension were put in 12 × 75 mm test tubes in a 37 °C water bath and were preincubated with 10 mM glucose for 5 min prior to addition of radioactive substrate. At 1-min intervals, a tube was removed from the bath, 1 mL of buffer A was added, and spheroplasts were collected and washed with 1 mL buffer A on a cellulose acetate filter (0.5-μm pore size) which had been prewetted with buffer A. Glutamine binding protein, which is quantitatively retained by nitrocellulose filters, completely passes through cellulose acetate filters. Filters were dried and counted in a 4 mL of Omnifluor (New England Nuclear) in toluene. Background was taken to be the number of counts retained by the filter at 0 min, with dilution by buffer A preceding addition of radioactive substrate. When included in assays, monopotassium arsenate, at a final concentration of 600 μM, was added at the start of the preincubation.

Transport by whole cells was assayed in a manner identical to that used for spheroplasts, using cells which had been grown, harvested, and washed exactly as for spheroplast preparation and finally resuspended in buffer A containing 100 μg/mL chloramphenicol. Transport by membrane vesicles was assayed in a similar manner. A volume of vesicles at 6–10 mg/mL protein in 0.1 M potassium phosphate, pH 6.6 (plus various additional small molecular weight compounds), was added to an equal volume of either 10 mM Tris-HCl, pH 7.0, or binding protein in 10 mM Tris-HCl, pH 7.0. $MgSO_4$ was added to 2 mM, the suspension was divided into 100-μL aliquots, and uptake of radioactive substrate was assayed as described above with the following changes: assays were performed at 28 °C, preincubations were for 10 min, and 2-mL volumes of 0.1 M LiCl were used to quench and wash vesicles (Kaback & Milner, 1970). The radioactive substrates used were L-[U-^{14}C]glutamine (3.7 mCi/mmol) at a final concentration of 34.5 μM or L-[3,4-$^3H_2$]glutamine (25 mCi/mmol) at a final concentration of 36.5 μM and L-[U-^{14}C]proline (6.1 mCi/mmol) at a final concentration of 39.4 μM.

Protein Determinations. Protein was determined by the method of Lowry (Lowry et al., 1951) with bovine serum albumin as the standard.

Results

Reconstitution Experiments with Spheroplasts. Cells of Gram-negative bacteria which have undergone disruption of the lipopolysaccharide layer of the cell wall by treatment with EDTA at alkaline pH, as well as partial hydrolysis of the peptidoglycan by lysozyme, are called spheroplasts (Kaback, 1971; Weiss, 1976). Contrary to their nomenclature, spheroplasts are usually not spherical. They are, however, very osmotically fragile and are readily lysed by violent mechanical agitation or upon dilution from 20% sucrose into hypotonic solutions. As was noted also by Galloway and Furlong with the ribose transport system, the major difficulty involved in spheroplast reconstitution experiments was the consistent preparation of spheroplasts (Galloway & Furlong, 1979). With our strains, the procedure described above reproducibly converted whole cells to stable spheroplasts if no alterations were made in the growth medium, the state at which cells were harvested, the concentration of lysozyme used, or the duration or temperature at which lysozyme treatment was carried out.

A routine reconstitution experiment with spheroplasts thus prepared from strain PSM2 is shown in Figure 1. The final concentration of added glutamine binding protein in this experiment was 0.68 mg/mL. Stimulation of glutamine transport was typically seen to increase with the concentration of added binding protein (data not shown) and reached a maximum of a 2.0–2.5-fold enhancement at an added binding protein concentration of 0.6–0.8 mg/mL. In order to establish that the apparent increased glutamine uptake of the sort depicted in Figure 1 was, indeed, due to the binding protein dependent active transport, it was necessary to show that it satisfied two conditions: first, that it was true active transport not dependent on the metabolizing of substrate and, second, that it was not merely due to a nonspecific interaction of liganded binding protein with spheroplasts or with the filters used in the transport assay.

The fulfillment of the first criterion was demonstrated with metabolic inhibitors. Chloramphenicol, an inhibitor of protein synthesis, was routinely included in all transport assays. The metabolism of glutamine, however, is highly divergent. Besides

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DNase, deoxyribonuclease; RNase, ribonuclease; azaserine, *O*-diazoacetyl-L-serine.

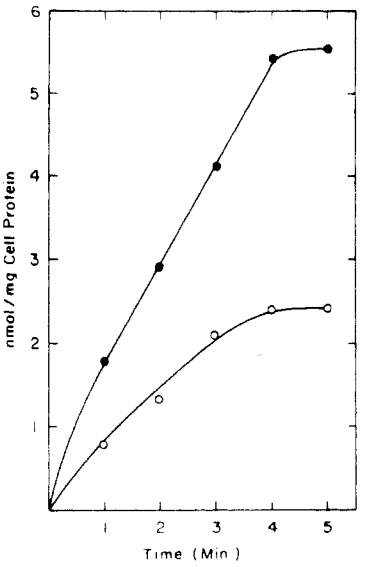

FIGURE 1: Glutamine transport by spheroplasts of strain PSM2 in the absence (○) and presence (●) of added purified glutamine binding protein to 0.68 mg/mL.

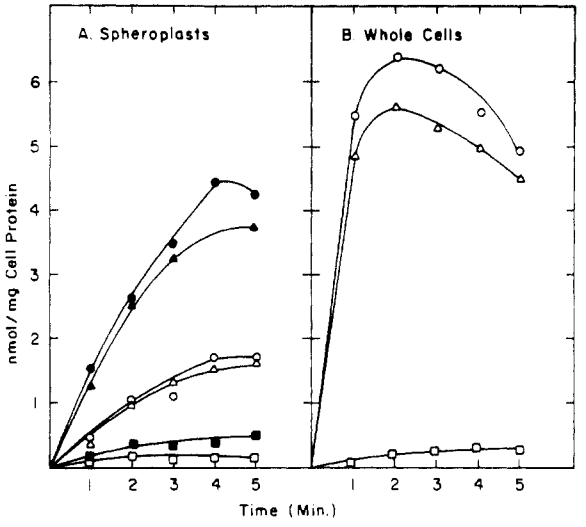

FIGURE 2: Effects of azaserine and arsenate on glutamine transport by spheroplasts and whole cells of PSM2. (A) Transport by spheroplasts: in the absence of added purified glutamine binding protein without arsenate or azaserine (○), with arsenate (□), or with azaserine (△); in the presence of added purified glutamine binding protein (0.79 mg/mL) without azaserine or arsenate (●), with arsenate (■), or with azaserine (▲). (B) Transport by whole cells: without arsenate or azaserine (○), with arsenate (□), or with azaserine (△).

being incorporated into protein, this amino acid plays a crucial role in the cell as a nitrogen donor in the biosynthesis of purines, pyrimidines, nicotinamide, carbamoyl phosphate, glucosamine phosphate, *p*-aminobenzoic acid, and other compounds (Meister, 1965). Many of these γ-glutamyl transfer reactions are inhibited by the glutamine analogue azaserine (*O*-diazoacetyl-L-serine) (Hartman, 1963; Levenberg et al., 1956; French et al., 1963). Weiner and Heppel, in their study of the glutamine binding protein, showed that when intact cells transported 6 μM glutamine, virtually all of it was rapidly converted to glutamate (Weiner & Heppel, 1971). When cells were incubated with 600 μM azaserine, these investigators saw no effect on the initial rate or steady-state level of glutamine uptake, and 85% of the transported substrate remained as free glutamine. As shown in Figure 2A, 600 μM azaserine had little effect on the transport of glutamine by spheroplasts in the presence or absence of added glutamine binding protein. The effect of the same concentration of azaserine on glutamine

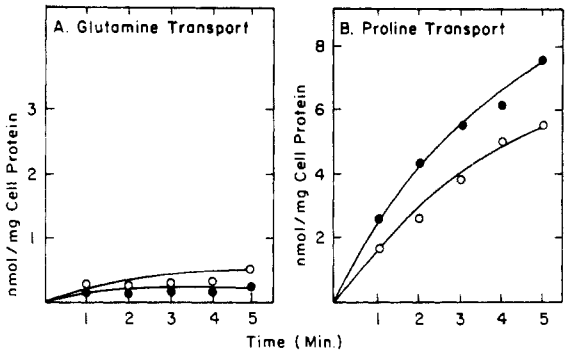

FIGURE 3: Transport of glutamine and proline by spheroplasts of PSM223 (*glnP* deletion) and PSM2. (A) Glutamine transport by spheroplasts of PSM223 in the absence (○) and presence (●) of added purified glutamine binding protein to 0.76 mg/mL. (B) Proline transport by spheroplasts of PSM223 (○) and PSM2 (●) in the absence of glutamine binding protein.

transport by whole cells is shown, for comparison, in Figure 2B. Azaserine also has no effect on the binding of glutamine by glutamine binding protein (Weiner & Heppel, 1971; our data, not shown). This compound is probably transported into cells by the shock-resistant, general aromatic amino acid transport system, as it is in *Salmonella* (Ames, 1964).

Two separate controls were used to show that the binding protein induced uptake of glutamine by spheroplasts was not artifactual. It should be noted that the filters used in spheroplast transport assays (cellulose acetate, 0.5-μm pore size) were completely permeable to binding protein. Counts retained by these filters after passage of binding protein plus labeled glutamine were always less than or equal to background counts. Nevertheless, further evidence was required to preclude the possibility that the increase of apparent transport by spheroplasts upon addition of binding protein was the result of liganded binding protein sticking to the spheroplasts, although such an interaction would have to have been time dependent. That this was not the case was shown directly by the total inhibiton of binding protein dependent glutamine transport in spheroplasts by 200 μM arsenate (Figure 2A), the classical inhibitor of osmotic shock sensitive active transport (Berger, 1973; Berger & Heppel, 1974). Arsenate inhibition of glutamine transport in intact cells is shown in Figure 2B. Furthermore, arsenate does not at all affect the binding of glutamine by glutamine binding protein (data not shown). A comparison of parts A and B of Figure 2 also shows that the addition of binding protein to spheroplasts restored glutamine transport to 70–80% of the level seen in whole cells.

Further proof of the specificity of the binding protein interaction with spheroplasts was obtained with PSM223, a strain having a deletion of the genes for the high-affinity glutamine transport system. Figure 3A shows that glutamine binding protein, when added to spheroplasts prepared from this strain, produced no increase in glutamine transport. That the spheroplasts used in this experiment were viable was shown by their ability to transport proline, which was comparable to that of spheroplasts from strain PSM2 (Figure 3B). Proline is taken up by a binding protein independent transport system (Berger, 1973; Berger & Heppel, 1974). This experiment, then, reinforced the results obtained by arsenate inhibition of PSM2. A second conclusion suggested by the failure of glutamine binding protein to restore transport in spheroplasts of PSM223 is that there is at least one additional gene linked to *glnP*, the gene for the binding protein, which codes for an essential component of glutamine transport and that this component is probably bound to the cytoplasmic membrane. If such a component did not exist, then one would expect

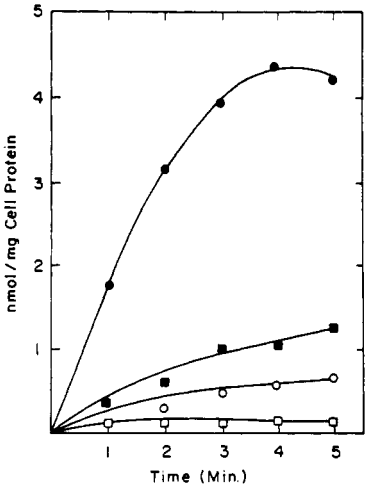

FIGURE 4: Transport of glutamine by spheroplasts of PSM116 (binding protein point mutant) and the effect of arsenate: in the absence of added purified glutamine binding protein without arsenate (○) or with arsenate (□); in the presence of added purified glutamine binding protein (0.69 mg/mL) without arsenate (●) or with arsenate (■).

spheroplasts prepared from PSM2 and PSM223 to be functionally equivalent with respect to their ability to interact with the glutamine binding protein.

In the reconstitution experiments with spheroplasts prepared from strain PSM2, there was a considerable background level of glutamine transport by spheroplasts in the absence of added glutamine binding protein (Figures 1 and 2A). That this transport was arsenate inhibitable (Figure 2A) implied that it was mainly due to glutamine binding protein not released upon formation of spheroplasts either because a fraction of the cell population remained resistant to lysozyme or because some domains of each cell envelope remained sufficiently intact to retain periplasmic proteins. This background level of glutamine transport was largely absent in spheroplasts prepared from strain PSM116, a derivative of PSM2 which has a point mutation in the structural gene for the glutamine binding protein (Figure 4). PSM116 produces amounts of glutamine binding protein equal to that produced by PSM2 and having the same molecular weight and antigenic cross-reactivity, but the mutant binding protein is completely unable to bind glutamine. When wild-type glutamine binding protein (from PSM2) was added to spheroplasts of strain PSM116, this mutant acquired the ability to transport glutamine as efficiently as spheroplasts of strain PSM2. Because of the low background level of glutamine transport in these spheroplasts, the extent of reconstitution was more than 6-fold. These results show that the mutation in PSM116 affects only the binding protein and does not exert a polar effect on the other component(s) of the glutamine transport system. As with PSM2, the binding protein dependent transport of glutamine by spheroplasts of PSM116 was inhibited by arsenate. However, a not insignificant fraction of binding protein dependent transport appeared to remain resistant to arsenate, which had not been the case with PSM2. This may have been due to aggregation of liganded binding protein in the assay, since when this experiment was performed, the purified glutamine binding protein had been stored in liquid nitrogen for 5 months. It has been observed by others that although glutamine binding protein remains active with respect to its ability to bind ligand, long-term storage of the protein results in changes in the proton magnetic resonance spectrum indicative of aggregation (Kreishman et al., 1973).

Experiments with Vesicles. Attempts were made to reconstitute binding protein dependent glutamine transport in *E. coli* membrane vesicles prepared by the method of Kaback (Kaback, 1971). A successful reconstitution was not obtained in spite of the incorporation into the vesicles of small molecular weight compounds as potential energy sources or effectors. Among the compounds incorporated, in various combinations in many independent experiments, were acetyl phosphate, acetyl coenzyme A, adenosine 5′-triphosphate, phosphoenolpyruvate, diphosphopyridine nucleotide (oxidized and reduced), triphosphopyridine nucleotide (oxidized and reduced), succinate, flavin adenine dinucleotide *S*-adenosylmethionine, adenosine 3′,5′-monophosphate, 3-phosphoglyceric acid, and oxaloacetic acid, in concentrations ranging from 200 μM to 10 mM. None of these were seen to stimulate binding protein dependent active transport of glutamine.

Discussion

The notion that the periplasmic binding proteins are essential components of particular active transport systems in *E. coli* and *S. typhimurium* has become well established since the discovery of this class of proteins some 15 years ago. The best evidence for this concept is genetic, but comparisons of the affinities, specificities, and conditions of induction or repression of given binding proteins and transport systems have been used as criteria for their association. In addition, the location of the binding proteins, their lack of any known enzyme activities, and the loss of certain types of transport upon their release from cells have also been taken as suggesting a role for these proteins in active transport. In particular, glutamine transport in *E. coli* appears to depend upon a periplasmic binding protein, the loss of which, either by genetic mutation or by osmotic shock, is concomitant with the loss of transport activity (Weiner & Heppel, 1971; unpublished results). Growth of cells in complex media represses glutamine transport and synthesis of glutamine binding protein. The binding protein and the transport system have a similar dissociation constant and apparent Michaelis constant for glutamine, and the glutamine analogues γ-glutamylhydrazide and γ-glutamylhydroxamate both competively inhibit binding and transport while other glutamine analogues, azaserine and 6-diazo-5-oxo-L-norleucine, inhibit neither binding nor transport (Weiner & Heppel, 1971). Upon binding ligand, the glutamine binding protein undergoes a marked conformational change detectable by nuclear magnetic resonance (Kreishman et al., 1973), fluorescence spectroscopy (Weiner & Heppel, 1971), or microcalorimetry (Marty et al., 1979), and this has been proposed to play a role in the transport process. As discussed by Pardee, individually, each of these pieces of evidence has limited value as a test of the association of the glutamine binding protein with the glutamine transport system (Pardee, 1970); however, taken together they strongly suggest such an association.

The data presented in this paper unequivocally establish that the glutamine binding protein is an essential component of the glutamine transport system. Whole cells of *E. coli* lose more than 60% of their ability to transport glutamine upon conversion to spheroplasts, and they gain most of this back upon addition of highly purified glutamine binding protein. The degree of reconstitution is even more dramatic in spheroplasts prepared from a strain unable to transport glutamine due to a mutation in the glutamine binding protein. The restored transport system is energy dependent, as shown by its sensitivity to arsenate, and it does not depend on the trapping of glutamine by metabolism, as evidenced by its insensitivity to azaserine. Furthermore, purified binding protein cannot restore glutamine transport in viable spheroplasts derived from a mutant strain having a deletion of the glutamine transport genes, indicating that the binding protein must interact with

at least one additional component of the transport system which is presumably located in the cytoplasmic membrane.

There have been two prior reports of reconstitution of binding protein dependent transport in spheroplasts of *E. coli*. In the first of these, osmotic shock sensitive phosphate transport was shown to be restored in spheroplasts by the addition of purified phosphate binding protein (Gerdes et al., 1977). This study had many difficulties, however. Cells had to be starved for phosphate before any transport could be assayed, and uptake of phosphate was dependent upon its intracellular esterification. Data was presented as rate of uptake vs. time, obscuring the fact that the kinetics of uptake had upward concavity, which is not indicative of a saturable transport system. In addition, arsenate inhibition of this system could not be demonstrated because it was necessary to remove by mutation the shock-resistant component of phosphate transport, which also transports arsenate. Galloway and Furlong did a very rigorous study of the reconstitution of binding protein-dependent ribose transport in spheroplasts (Galloway & Furlong, 1979). These authors showed that the degree of reconstitution was proportional to the concentration of added binding protein, reaching a maximum at 1 mg/mL. Restoration of transport by binding protein was dependent upon the induction of the ribose transport system, but it did not require the metabolizing of ribose since reconstitution could be achieved with a strain that was constitutive for the transport system but lacked ribokinase. In recent work with this system, it was shown that binding protein dependent transport could be restored in spheroplasts from a mutant strain which is defective in the ribose binding protein but normal in the other component(s) of the ribose transport system (Robb et al., 1980).

A principal motivation behind the study presented in this paper was to achieve a first step in the development of a membrane vesicle system for the assay of osmotic shock sensitive transport. Such a system remains an elusive goal. No binding protein dependent transport of glutamine could be detected in numerous vesicle preparations in which were incorporated various possible energy donor molecules. The most likely reason for this failure to reconstitute is that the correct energy source for osmotic shock sensitive transport has yet to be found. Other explanations are conceivable, though. It may be that one or more cytoplasmic macromolecular components are required for glutamine transport or, equivalently, that required components peripherally bound to the membrane are stripped away during the EDTA washes involved in the preparation of vesicles. Less "clean" vesicle preparation procedures may have to be devised in order to preserve the integrity of shock-sensitive transport components. This work ought to be pursued further since the development of a vesicle system in which binding protein dependent transport can be assayed is a prerequisite for serious mechanistic studies of the osmotic shock sensitive transport systems.

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