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Properties and Characterization of Binding Protein Dependent Active Transport of Glutamine in Isolated Membrane Vesicles of *Escherichia coli*†

Arthur G. Hunt‡ and Jen-shiang Hong*

ABSTRACT: The reconstituted binding protein dependent active transport of glutamine in isolated membrane vesicles of *Escherichia coli* [Hunt, A. G., & Hong, J. (1981) *J. Biol. Chem. 256*, 11988-11991] is characterized in some detail. Transport activity exhibits a rather narrow pH optimum at about 5.8 with apparent pK_as of 5.3 and 6.6, is inhibited by increasing ionic strength, and requires potassium and phosphate ions. However, the binding of glutamine to the glutamine binding protein is unaffected by pH over a range of 5-8, is relatively insensitive to variation in ionic strength up to 1.0 M KCl, and does not require potassium and phosphate ions. Since the internal pH of vesicles does not change over the range of 5-8, the pH-dependent transport profile most probably reflects the interaction of liganded glutamine binding protein with the membrane-bound components of the glutamine transport system. Two classes of compounds can serve as exogenous sources of energy for glutamine transport. One class consists of those compounds that can be metabolized to pyruvate. This class of compounds is effective only if NAD is incorporated into vesicles, and only if vesicles are prepared from strains containing active phosphotransacetylase and acetate kinase. The second class of compounds, of which succinate is the sole member, is effective in vesicles containing only those small molecules present in the lysis buffer, and in vesicles prepared from phosphotransacetylase and acetate kinase mutant strains as well as from the parent PSM116. It appears that succinate or a yet to be determined metabolite derived from succinate or a common product of pyruvate and succinate metabolism is the energy donor for glutamine transport. ATP and/or acetyl phosphate are found to be inactive as a source of energy in vesicles. Substances that abolish the electrochemical proton gradient ($\Delta\bar{\mu}_H$), either by conducting protons across the vesicular membrane or by halting respiration, inhibit glutamine transport in vesicles. However, it appears that $\Delta\bar{\mu}_{H^+}$ is required for glutamine transport for a role other than serving as an energy donor as in the $\Delta\bar{\mu}_{H^+}$-driven shock-resistant transport. The membrane vesicle preparations described here possess considerable metabolic capabilities. Vesicles are capable of incorporating ^{32}P from P_i into ATP, ADP, AMP, GTP, acetyl phosphate, and several unidentified phosphate-containing compounds, indicating the presence of pyruvate dehydrogenase, phosphotransacetylase, acetate kinase, Mg^{2+}-ATPase, adenylate kinase, nucleoside (AMP) phosphatase, nucleotide (adenosine) kinase, and nucleoside diphosphate kinase in vesicles.

The glutamine transport system of *Escherichia coli* is one of those whose activity is abolished by a cold osmotic shock treatment of whole cells [for a review, see Wilson (1978)]. Shock-sensitive transport systems possess this unique property because of the involvement of soluble, periplasmic substrate binding proteins in transport. Osmotic shock releases these proteins from cells, rendering these transport systems inactive. Shock-resistant transport systems, on the other hand, require only cytoplasmic membrane-bound components for their activity and are therefore not affected by the loss of periplasmic proteins.

The involvement of the glutamine binding protein in glutamine transport has been firmly established. This protein is seen to bind glutamine with an affinity for glutamine very similar to that of transport in whole cells (Weiner & Heppel, 1971). Removal of this binding protein, by either osmotic shock (Weiner & Heppel, 1971), mutation (Masters & Hong, 1981a), or lypozyme-ethylenediaminetetraacetic acid (EDTA)[1] treatment (Masters & Hong, 1981b), greatly diminishes glutamine transport. Moreover, addition of purified glutamine binding protein to spheroplasts restores the glutamine transport ability of spheroplasts (Masters & Hong, 1981b).

Very little is known about the membrane-bound components of the glutamine transport system of *E. coli*. The existence of these components has been proven by Masters & Hong (1981b), but their number and properties are not established.

† From the Department of Cell Physiology, Boston Biomedical Research Institute, Boston, Massachusetts 02114. *Received August 13, 1982.* This work was supported by Grants GM22576 and GM29843 from the National Institute of General Medical Sciences.

‡ Present address: Laboratory of Plant Molecular Biology, The Rockefeller University, New York, NY 10021.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; $\Delta\bar{\mu}_{H^+}$, electrochemical proton gradient; $\Delta\psi$, electrical potential; ΔpH, chemical gradient of hydrogen ions; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; *ack*, acetate kinase; *pta*, phosphotransacetylase; DON, 6-diazo-5-oxo-L-norleucine; PEP, phosphoenolpyruvate; CoA, coenzyme A; Mops, 4-morpholinepropanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PEI, poly(ethylenimine).

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Table I: Bacterial Strains

strain	genotype	parent	comments
PSM2	F⁻ *thi metC glnP$_O$*		Masters & Hong (1981a)
PSM116	F⁻ *thi metC glnP$_O$*	PSM2	Masters & Hong (1981a)
AGH40	F⁻ *thi metC glnp$_O$*	PSM116	spontaneous, fluoroacetate resistant
AGH41	F⁻ *thi metC glnp$_O$ glnP ack*	PSM116	spontaneous, fluoroacetate resistant
AGH39	F⁻ *thi metC glnP$_O$ glnP il*	PSM116	by diethyl sulfate mutagenesis, cycloserine enrichment
PSM39	F⁻ *thi metC uncA*		
AGH42	F⁻ *thi metC glnP$_O$ glnP uncA*	AGH39	by transduction with P_1 lysate of PSM39

Preliminary data obtained by using recombinant DNA techniques indicate that the glutamine transport operon encodes several different polypeptides, one of which is the glutamine binding protein.[2]

Also unknown is the nature of the interaction of the glutamine binding protein with the membrane-bound components of the glutamine transport system. Studies concerning this interaction have been hindered by the lack of an appropiate system in which to study them. Recently, we described the successful reconstitution of binding protein dependent glutamine transport in isolated *E. coli* membrane vesicles (Hunt & Hong, 1981). Here, we present a detailed characterization of this system and compare the properties of glutamine transport in vesicles with those of glutamine binding by its binding protein. We also discuss the involvement of $\Delta\bar{\mu}_{H^+}$ in glutamine transport and address the nature of the energetics of glutamine transport in vesicles.

Materials and Methods

Materials. All radioactive compounds were obtained from New England Nuclear. Nitrocellulose filters used for the assay of glutamine binding protein were from Schleicher & Schuell. Cellulose acetate filters used for the transport assays were from Millipore or Schleicher & Schuell. PEI–cellulose precoated TLC plates were from Brinkmann. NAD, CoA, and ADP were from Boehringer-Mannheim. ATP, AMP, GTP, DON, *S*-carbamyl-L-cysteine, albizzine, methionine sulfoximine, azaserine, D-glutamine, sodium azide, and CCCP were from Sigma. γ-Glutamyl hydrazide was from ICN. All other chemicals were reagent grade and obtained from commercial sources.

Strains and Media. Six derivatives of *E. coli* K12 were used (Table I). All of these strains were derived from PSM2 (*thi metC glnP$_O$*), a strain harboring a regulatory mutation that causes an overproduction of the glutamine transport system (Masters & Hong, 1981a). A plasmid containing the coding sequence for the phosphoglycerate transport system of *Salmonella typhimurium* (pJH7) was used to confer upon strains the ability to take up PEP. The isolation of this plasmid will be described elsewhere.

Mineral medium E (Vogel & Bonner, 1956), containing 0.5% carbon source, 0.4 mM methionine, and 40 μM vitamin B_1, was the medium used for cell growth in the experiments described here.

Purification of the Glutamine Binding Protein. Glutamine binding protein was purified from succinate-grown PSM2 as described previously (Hunt & Hong, 1981) and stored frozen in liquid nitrogen.

Preparation of Isolated Membrane Vesicles. Isolated membrane vesicles were prepared from glycerol-grown cells as described previously (Hunt & Hong, 1981). Except where indicated, NAD (0.1 mM), ADP (0.1 mM), and CoA (10 μM) were included in the lysis buffer. [The NAD requirement is discussed here and elsewhere (Hunt & Hong, 1981). The addition of CoA and ADP reflects the requirement for phosphotransacetylase and acetate kinase discussed in this paper. Although the incorporation of these latter compounds is somewhat dispensable, their presence makes results from preparation to preparation more reproducible and may enhance the stability of the glutamine transport activity of vesicles.] In some instances, sodium phosphate replaced potassium phosphate in the lysis buffer. Phosphate-free lysis buffer consisted of 50 mM Mops adjusted to pH 7.0 with KOH.

PSM2 spheroplasts were washed, where indicated, by repeated resuspension in Tris-HCl, pH 8.0, + 100 μg/mL chloramphenicol before lysis as described elsewhere (Hunt & Hong, 1981).

Vesicles were stored frozen in liquid nitrogen. The succinate-driven glutamine transport ability of vesicles proved to be stable for at least 4 weeks, whereas the pyruvate-driven transport ability decreased by about 50% after 1 week, as noted earlier (Hunt & Hong, 1981). This instability may reflect the demonstrated cold lability of acetate kinase (Anthony & Spector, 1971), an enzyme apparently required for pyruvate-driven transport (see below).

Glutamine Transport Assays. Glutamine transport in vesicles was assayed in the presence of saturating glutamine concentrations as described previously (Hunt & Hong, 1981), except that the final pH was, in most cases, brought to 6.1 by the addition of 1 μL of 1.0 M KH_2PO_4 for every 50 μL of the vesicle–binding protein mixture. Additions were made as detailed in the text. For determination of the pH–activity profile, equal volumes of the vesicle–binding protein mixture, containing $MgSO_4$ and the exogenous energy source, and H_3PO_4–KOH solutions were mixed, the pH of the suspension was measured, and 100-μL aliquots were assayed for glutamine transport by the usual assay. Likewise, the effect of ionic strength was examined by mixing equal volumes of the vesicle–binding protein mixture (at pH 6.1) and solutions of KCl, so that the final concentration of KCl was that designated in the text. A 100-μL sample was then assayed for transport as described (Hunt & Hong, 1981). Transport was assayed in all instances at 37 °C. The glutamine concentration in all cases was 36.4 μM.

Assay of Glutamine Binding Activity. The binding activity of the glutamine binding protein was assayed at a saturating concentration of glutamine (13.4 μM) by a filter binding assay described previously (Masters & Hong, 1981b; Hunt & Hong, 1981), except that when indicated, the carbon-free salts medium routinely used was replaced with 0.15 M potassium (or, in one instance, sodium) phosphate, pH 7.0, or 15 mM Tris-HCl, pH 7.0, when a phosphate-free buffer was called for. Additions were made to these assays as indicated in the text.

Examination of Phosphate-Containing Compounds in Vesicles. Vesicles, binding protein, and $MgSO_4$ were mixed as described for transport assays, and ^{32}P (as inorganic phosphate, in HCl-free water) was added to a final specific activity of 1–10 mCi/mmol. Aliquots (50 μL) of this mixture

[2] J. Hong, unpublished experiments.

Table II: Ability of Various Substances To Serve as Exogenous Energy Sources for Glutamine Transport in Vesicles[a]

compound	rate of glutamine transport [nmol (mg of vesicle protein)$^{-1}$ min^{-1}]	stimulation (x-fold over control)
none	0.012	1
pyruvate	0.205	17.1
D-lactate	0.257	21.4
succinate	0.534	44.5
fumarate	0.008	
malate	0.003	
oxaloacetate	0.006	
glucose	0.011	
glucose 6-phosphate	0.012	
acetate	0.002	
acetyl phosphate	0.004	

[a] Transport assays were performed as described under Materials and Methods. Exogenous energy sources were added to 20 mM and vesicles preincubated for 10 min at 37 °C. Succinate, fumarate, malate, and oxaloacetate solutions were neutralized with KOH before use; other compounds were added as sodium salts.

were then incubated at 37 °C for 15 min in the presence or absence of an exogenous energy source. These aliquots were then diluted and filtered as described for transport assays, and the filter was immediately extracted with 1.0 M formic acid. Duplicate filters were usually extracted into 0.25 mL of formic acid. These extracts were stored frozen at −20 °C.

Extracts were analyzed by thin-layer chromatography (TLC) on PEI–cellulose; chromatograms were developed in 1.5 M potassium phosphate, pH 3.4, or 1.0 M LiCl. TLC plates were dried under a stream of hot air and analyzed by autoradiography, using Kodak X-omat X-ray film. Compounds were identified, where possible, by comparison with known standards whose location had been determined under a short-wavelength ultraviolet lamp.

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

Results

Compounds Capable of Driving Glutamine Transport in Isolated Membrane Vesicles. Previously, we demonstrated that membrane vesicles prepared from a strain of *E. coli* producing defective glutamine binding protein, when possessing incorporated NAD, were capable of using exogenous pyruvate or D-lactate to drive binding protein dependent glutamine transport (Hunt & Hong, 1981). Further experiments showed that, in addition to these compounds, vesicles were also able to use succinate to drive glutamine transport (Table II). Fumarate, malate, and oxaloacetate were all ineffective in these vesicles. Furthermore, incorporated NAD, though somewhat stimulatory, was not required for succinate-driven glutamine transport in vesicles (Figure 1). This is in contrast to what has been shown for pyruvate- or D-lactate-driven glutamine transport (Hunt & Hong, 1981) and allows a classification of exogenous energy sources for glutamine transport into the following two groups: (1) those compounds that are metabolically related to pyruvate (pyruvate, D-lactate, and, in addition, phosphoenolpyruvate), when vesicles capable of transporting this compound are studied (Hugenholtz et al., 1981; see below), and that require incorporated NAD for their effect; (2) succinate.

Effect of pH and Ionic Variations on Glutamine Transport in Vesicles and on Glutamine Binding by the Glutamine

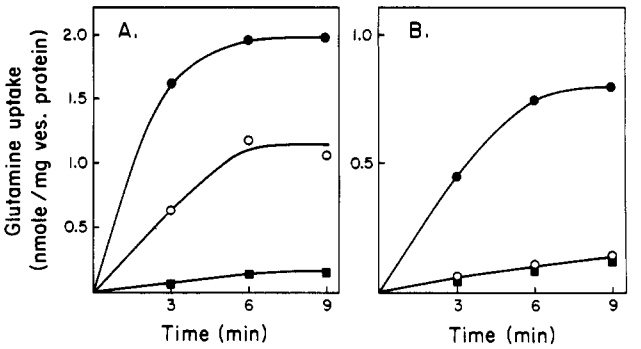

FIGURE 1: Glutamine transport properties of vesicles prepared with and without incorporated NAD. Vesicles, prepared as described under Materials and Methods, were assayed for glutamine transport in the presence of 0.25 mg/mL glutamine binding protein as described under Materials and Methods. (A) Vesicles containing 0.1 mM NAD: (●) +20 mM succinate; (○) +20 mM pyruvate; (■) no added energy source. (B) Vesicles with no additional compounds incorporated into them: (●) +20 mM succinate; (○) +20 mM pyruvate; (■) no added energy source.

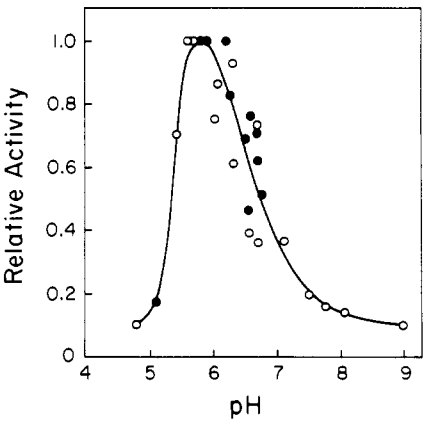

FIGURE 2: pH–activity profile of glutamine transport in vesicles. Vesicles were assayed for glutamine transport in the presence of 0.30 or 0.83 mg/mL glutamine binding protein at various pHs as described under Materials and Methods. Three-minute uptake was measured, and the data were normalized against the largest value. (●) 20 mM succinate as the exogenous energy source; (○) 20 mM pyruvate as the exogenous energy source.

Table III: Glutamine Binding Properties of the Glutamine Binding Protein[a]

treatment	nmol bound	% of control
none	0.0632	100
NaP_i buffer	0.0743	118
KP_i buffer, pH 5.0	0.0515	82
KP_i buffer, pH 6.0	0.0646	102
KP_i buffer, pH 8.0	0.0631	100
+0.5 M KCl	0.0598	95
+0.75 M KCl	0.0525	83
Tris buffer (phosphate free)	0.0492	78
+γ-glutamyl hydrazide	0.0068	11
+6-diazo-5-oxo-L-norleucine	0.0637	101
+5-carbamyl-L-cysteine	0.0444	70
+albizziine	0.0621	98
+L-methionine DL-sulfoximine	0.0667	106
+D-glutamine	0.0602	95
+L-glutamine (unlabeled)	0	0

[a] Glutamine binding protein (4.3 μg) was assayed for glutamine binding as described under Materials and Methods, using 0.15 M KP_i, pH 7.0, in place of the carbon-free salts medium. This buffer was replaced, where indicated, by 0.15 M NaP_i, pH 7.0, 15 mM Tris-HCl, pH 7.0, or 0.15 M KP_i of the pH indicated. Glutamine analogues were added to 2 mM. Other additions were as indicated.

Table IV: Properties of Glutamine Transport in Membrane Vesicles[a]

treatment	initial rate of transport [nmol min^{-1} (mg of vesicle protein)$^{-1}$]	% of control
none	0.54	100
+0.25 M KCl	0.54	100
+0.50 M KCl	0.26	49
+0.75 M KCl	0.02	4
+γ-glutamyl hydrazide	0.20	38
+6-diazo-5-oxo-L-norleucine	0.99	184
+5-carbamyl-L-cysteine	0.36	67
+albizziine	0.53	100
+L-methionine DL-sulfoximine	0.83	153
+D-glutamine	0.70	130
+L-glutamine (unlabeled)	0.03	6
NaP_i (instead of KP_i)	0.06	11

[a] Transport in vesicles was assayed as described under Materials and Methods. In one instance, vesicles were prepared and assayed in NaP_i buffer instead of KP_i buffer. Assays in the presence of KCl were performed as described under Materials and Methods. Glutamine analogues were added to a final concentration of 2 mM. The final binding protein concentration, in all cases, was 0.21 mg/mL. Assay mixtures contained between 2.1 and 2.6 mg of vesicle protein per mL.

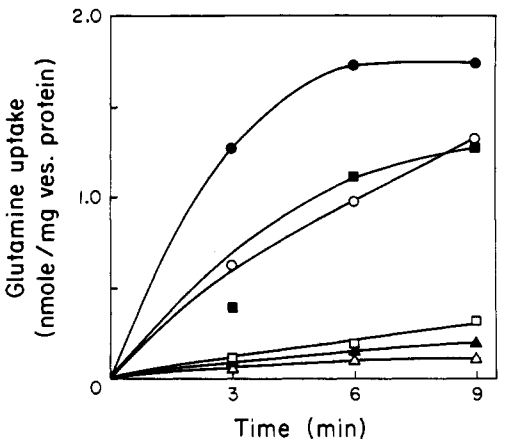

FIGURE 3: Requirement of inorganic phosphate for glutamine transport in vesicles. Vesicles, prepared in potassium phosphate (●, ■, ▲) or Mops (○, □, △) buffers as described under Materials and Methods, were assayed for glutamine transport in the presence of 0.25 mg/mL glutamine binding protein, as described under Materials and Methods. (●, ○) +20 mM succinate; (■, □) +20 mM D-lactate; (▲, △) no added energy source.

Binding Protein. Figure 2 shows the pH–activity profile of binding protein dependent glutamine transport in membrane vesicles. There is a relatively narrow range of optimal activity centered at a pH of 5.8, with distinct apparent pK_as of 5.3 and 6.6. In contrast, the binding of glutamine by glutamine binding protein is unchanged over a pH range of 5.0–8.0 (Table III).

Table IV summarizes the effects of variations in ionic strength and composition on glutamine transport. Glutamine transport was inhibited by increasing ionic strength, showing only 50% of control activity at 0.5 M KCl and virtually no activity at 0.75 M KCl. Replacement of potassium with sodium in the preparation and assay of vesicles abolished their ability to transport glutamine. Inorganic phosphate was essential for maximal activity but somewhat dispensable for succinate-driven transport (Figure 3).

These properties can be compared with the effects of similar variations on glutamine binding by its binding protein (Table III). Glutamine binding was relatively insensitive to variations in ionic strength from 0 to 1.0 M KCl, and in the ionic composition detailed above, and did not require inorganic phosphate.

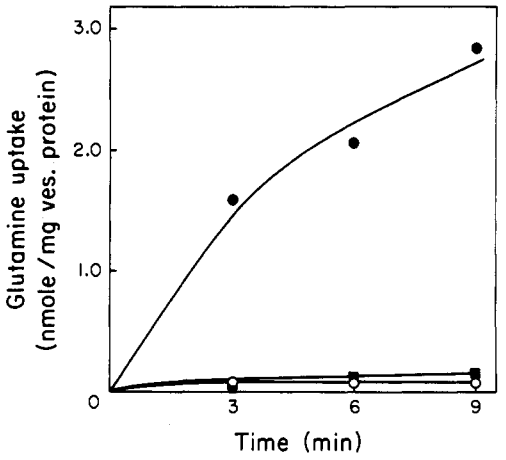

FIGURE 4: Effect of CCCP, KCN, and sodium azide on glutamine transport in vesicles. Vesicles were assayed for glutamine transport in the presence of 0.25 mg/mL glutamine binding protein as described under Materials and Methods. (●) +20 mM succinate; (○) +20 mM succinate + 2.5 μM CCCP, 5 mM KCN, or 2 mM sodium azide; (■) no additions.

Effect of Glutamine Analogues on Transport and Binding. Two analogues of glutamine, γ-glutamyl hydrazide and γ-glutamylhydroxamate, have been reported to inhibit glutamine transport in whole cells and the binding of glutamine to its binding protein (Weiner & Heppel, 1971). The results of a similar survey conducted with isolated membrane vesicles are summarized in Table IV. γ-Glutamyl hydrazide inhibited transport in the system described here, as well as the binding of glutamine to its binding protein, corroborating the results of Weiner and Heppel. *S*-Carbamyl-L-cysteine, a compound not tested by these authors, also inhibited both transport and binding. None of the other glutamine analogues tested, or the stereoisomer D-glutamine, had any inhibitory affect on transport or binding; indeed, some analogues actually enhanced glutamine transport to some extent.

Effects of Uncouplers and Inhibitors of Respiration on Glutamine Transport. Several investigators have suggested that an electrochemical gradient of protons ($\Delta\bar{\mu}_{H^+}$) is required for shock-sensitive transport in *E. coli* (Plate et al., 1974; Singh & Bragg, 1977, 1979; Plate, 1979). Therefore, we studied the effects of various agents known to diminish $\Delta\bar{\mu}_{H^+}$ on glutamine transport in isolated membrane vesicles (Figure 4). CCCP (a lipophilic weak acid that abolishes $\Delta\bar{\mu}_{H^+}$), potassium cyanide (an inhibitor of electron transport via the respiratory chain), and sodium azide (also an inhibitor of respiration) all completely abolished glutamine transport in membrane vesicles. This was true in the presence of high external concentrations of the exogenous energy source and in vesicles that had been preincubated for 10 min with succinate in the absence of inhibitor. These results agree with those seen by others and suggest that $\alpha\bar{\mu}_{H^+}$ is required for glutamine transport in vesicles.

Metabolic Capabilities of Isolated Membrane Vesicles. The direct energy donor for shock-sensitive transport in *E. coli* is not known (Hunt & Hong, 1982). High-energy phosphate is apparently required for transport, but the identity of the high-energy compound involved is not clear. The vesicle preparations described here are clearly capable of generating the required energy donor for shock-sensitive transport from pyruvate or succinate. This suggests that these vesicles are

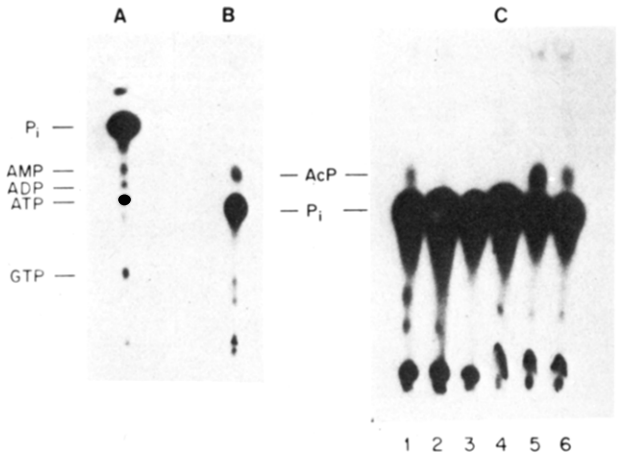

FIGURE 5: Phosphate-containing compounds made by vesicles from various strains. Membrane vesicles were labeled with ^{32}P and extracted, and the extracts were analyzed as described under Materials and Methods. For (A) and (B), vesicles from PSM116 were labeled at pH 7.0 in the presence of 20 mM pyruvate: (A) TLC developed in 1.5 M potassium phosphate, pH 3.4; (B) TLC developed in 1.0 M LiCl. For (C), vesicles from strains containing pJH7 plasmid were labeled at pH 6.1, and the TLC was developed in 1.0 M LiCl. Lane 1, pJH7/PSM116 + 20 mM PEP; lane 2, pJH7/PSM116, no PEP; lane 3, pJH7/AGH40 (*pta*) + 20 mM PEP; lane 4, pJH7/AGH40, no PEP; lane 5, pJH7/AGH41 (*ack*) + 20 mM PEP; lane 6, pJH7/AGH41, no PEP.

Table V: Transport Ability of Membrane Vesicles Prepared from Various Mutant Strains[a]

vesicle source	energy source	glutamine transport [nmol (mg of vesicle protein)$^{-1}$ (3 min)$^{-1}$]
wild type	pyruvate	0.62
AGH42 (*unc*)	pyruvate	1.10
AGH40 (*pta*)	succinate	0.95
	pyruvate	0.12
	none	0.06
AGH41 (*ack*)	succinate	0.71
	pyruvate	0.10
	none	0.06
pJH7/PSM116	PEP	2.0 (0.78)[b]
pJH7/AGH40	PEP	0.11 (0.31)
pJH7/AGH41	PEP	0.11 (0.48)

[a] Transport assays were performed as described under Materials and Methods. Exogenous energy sources were added to 20 mM and vesicles preincubated for 10 min at 37 °C. Experiments with AGH40 and AGH41 vesicles were done with 0.25 mg/mL glutamine binding protein at pH 7.0. [b] Numbers in parentheses are proline transport [nmol (mg of vesicle protein)$^{-1}$ (3 min)$^{-1}$].

capable of substantial metabolism. A survey of the phosphate-containing compounds produced by vesicles confirms this suspicion (Figure 5A,B). Among the compounds seen in vesicles, ATP, ADP, AMP, GTP, and acetyl phosphate could be identified. This was true of vesicle prepared as described under Materials and Methods, with incorporated NAD, CoA, and ADP, as well as of vesicles into which no cofactors or substrates had been incorporated, when given exogenous succinate (data not shown). This observation indicates that small amounts of cofactors, substrates, and soluble enzymes, amounts sufficient to give vesicles a modest metabolic capability, are trapped inside of vesicles during their preparation.

Transport Ability of Vesicles Prepared from Mutant Strains. In order to study the involvement of specific enzymes in the energization of glutamine transport in vesicles, we examined the transport properties of vesicles prepared from mutant strains. Vesicles prepared from a strain lacking the

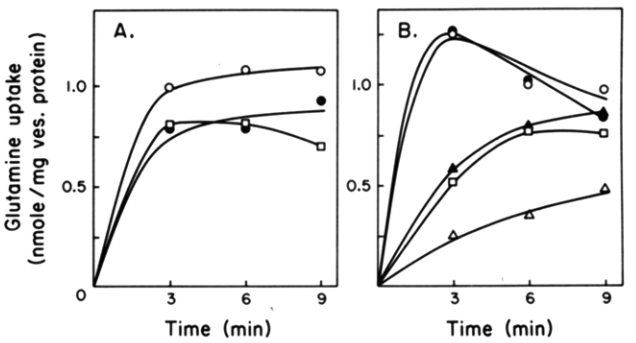

FIGURE 6: Glutamine uptake in vesicles from PSM2. Glutamine uptake into PSM2 vesicles was assayed as described under Materials and Methods, except assays were performed at pH 7.0. (A) uptake in the presence of 0.25 mg/mL glutamine binding protein: (●) +20 mM succinate; (○) +20 mM pyruvate; (□) no added energy source. (B) Uptake with no added binding protein: (●) +20 mM succinate; (○) +20 mM pyruvate; (□) no added energy source; (▲) +20 mM succinate + 5 mM arsenate; (△) +5 mM arsenate.

Mg^{2+}-ATPase transported glutamine at least as well as vesicles prepared from the wild-type parent (Table V). This result demonstrates that the H^+-transducing ATPase plays no role in the energization of glutamine transport in vesicles. In contrast, vesicles prepared from derivatives of PSM116 lacking either phosphotransacetylase or acetate kinase were unable to use pyruvate (or D-lactate) to drive glutamine transport but were able to use succinate for this purpose (Table V). In all of these different vesicle preparations, the D-lactate-driven transport of proline, via a $\Delta\bar{\mu}_{H^+}$-driven shock-resistant system (Ramos & Kaback, 1977a,b), was normal (data not shown). These results indicate that $\Delta\bar{\mu}_{H^+}$ does not drive glutamine transport in vesicles. Phosphotransacetylase and acetate kinase, on the other hand, seem to be involved in the derivation of the energy donor for glutamine transport from pyruvate or D-lactate, but not from succinate.

ATP and Acetyl Phosphate Are Insufficient To Drive Glutamine Transport in Vesicles. Previously, arguments suggesting roles for ATP (Berger, 1973; Berger & Heppel, 1974) and acetyl phosphate (Hong et al., 1979) in shock-sensitive transport have been presented. Using strains of *E. coli* harboring a plasmid containing the phosphoglycerate transport (pgt) system from *Salmonella typhimurium* (Hugenholtz et al., 1981), we tested the possibility that ATP and acetyl phosphate are sufficient to drive glutamine transport in vesicles. As shown in Table V, vesicles prepared from PSM116 harboring the *pgt* plasmid were able to use PEP to drive glutamine transport. However, vesicles prepared from the *ack* and *pta* strains containing the same plasmid were unable to use PEP for this purpose. PEP-driven proline transport in all three types of vesicles was substantial. This result indicates that ATP formation from ADP and PEP, via endogenous-trapped pyruvate kinase, occurs in the *ack* and *pta* vesicles, since PEP drives proline transport in vesicles via ATP formation and the subsequent formation of $\Delta\bar{\mu}_{H^+}$ upon hydrolysis of ATP by the Mg^{2+}-ATPase (Hugenholtz et al., 1981). Furthermore, ^{32}P labeling experiments shown in Figure 5C demonstrated that the *ack* vesicles also formed significant quantities of acetyl phosphate. This indicates that vesicles require more than just the ability to form ATP and acetyl phosphate in order to use PEP to drive glutamine transport.

Properties of Membrane Vesicles Prepared from PSM2. Previously, we showed that, in order to effect a successful reconstitution of binding protein dependent glutamine transport in vesicles, endogenous glutamine binding protein had to be removed from the appropriate strain by mutation (Hunt &

Hong, 1981). Although we showed that vesicles prepared from a strain that overproduces binding protein do in fact contain significant quantities of binding protein, the nature for the large amount of binding protein independent glutamine uptake seen in these vesicles remained obscure. In order to gain insight into the nature of the glutamine uptake seen in PSM2 vesicles, we performed a study of the properties of this uptake. As seen in Figure 6, this uptake was not stimulated by binding protein and only marginally stimulated by exogenous pyruvate or succinate. Incorporated NAD was not required for this uptake. Arsenate had only a modest effect on the uptake seen (Figure 6), in contrast to the inhibition seen with PSM116 vesicles (Hunt & Hong, 1981). Previously, attempts to wash the endogenous binding protein from PSM2 vesicles with high salt concentrations were unsuccessful (Hunt & Hong, 1981). Washing the spheroplasts from which these vesicles were derived with buffer of higher pH (in this case pH 8.0) was also without effect (data not shown). Exogenous glutamate and azaserine were without effect, as was incorporated γ-glutamyl hydrazide (data not shown). Finally, the apparent K_m for glutamine uptake in these vesicles was found to be greater than 2×10^{-6} M, a value almost 10 times greater than that reported for glutamine transport in whole cells (Weiner & Heppel, 1971) and PSM116 vesicles (Hunt & Hong, 1981). These results are difficult to interpret but may indicate some irreversible interaction of the glutamine binding protein of PSM2 with membrane vesicles, caused perhaps by the degree of overproduction seen in this strain (Masters & Hong, 1981a). This binding protein is probably responsible for the retention of glutamine by vesicles, thus explaining the lack of any energy dependence of this uptake. The localization of the endogenous binding protein (inside the vesicles, on the inner face of the membrane, or the outer face of the membrane) is unclear, although the high apparent K_m for glutamine in these vesicles suggests that it is located inside of the vesicles or on the inner face of the membrane.

Discussion

The results described in this paper have characterized in some detail a membrane vesicle preparation capable of glutamine transport in a binding protein stimulated manner. The characteristics of glutamine transport have been compared to those of glutamine binding to its binding protein. Furthermore, these results have addressed several questions concerning binding protein dependent glutamine transport in *E. coli* and underline the potential utility that this system holds for the study of this system.

The glutamine transport activity of membrane vesicles under the conditions described here reflects several processes: the binding of glutamine to its binding protein; the interaction of liganded binding protein to other, presumably membrane-bound components of the glutamine transport system; and the generation of the necessary energy donor for transport by vesicles. It is therefore instructive to compare the characteristics of glutamine transport presented here with the glutamine binding properties of the binding protein, as reported here and elsewhere, and with previously described properties of isolated *E. coli* membrane vesicles.

The pH profile of glutamine transport clearly does not reflect the ability of the glutamine binding protein to bind glutamine since this property is unaffected over a pH range of 5–8(Table III). Likewise, it cannot be explained by an inability of vesicles to take up succinate or pyruvate, since the ability of vesicles to take up substances via osmotic shock resistant, $\Delta\bar{\mu}_{H^+}$-driven transport systems is, in general, not affected over this pH range (Lombardi & Kaback, 1972). This is especially true of succinate uptake in vesicles (Rayman et al., 1972). Moreover, the internal pH of vesicles does not change dramatically over this pH range (Ramos et al., 1976; Ramos & Kaback, 1977a). Therefore, the pH profile most probably reflects the interaction of liganded glutamine binding protein with the membrane-bound components of the glutamine transport system. Indeed, in light of results presented elsewhere (Hunt & Hong, 1983) that show that the histidine-specific reagent diethyl pyrocarbonate affects the ability of the glutamine binding protein to restore transport in vesicles, it is tempting to speculate that the pK_a of 6.6 seen here for transport in vesicles may reflect the ionization of the sole histidine residue of the glutamine binding protein.

Interpreting the effects of changes in the ionic composition on the transport activity seen in vesicles is more difficult, although the results presented in Table III make it clear that these effects are not due to any changes in the ability of the glutamine binding protein to bind glutamine. The effect of increasing ionic strength on transport probably reflects a role of ionic interactions in the interaction of liganded binding protein with membranes but may also indicate a decreased ability of vesicles to take up and properly metabolize the exogenous energy source. Conversely, the potassium and phosphate requirements probably reflect specific requirements for the energization of vesicles for transport, although a possible involvement of one or more of these ions in the binding protein–membrane interaction cannot be ruled out.

The specificity of the glutamine transport seen in vesicles is clearly determined by the glutamine binding protein, as the results in Tables III and IV demonstrate. Weiner & Heppel (1971) have reached the same conclusion by using whole cells. The glutamine binding protein tolerates no changes in stereospecificity or in the carboxyl or α-amino moiety of the glutamine molecule. Furthermore, changes in the side chain are only poorly tolerated, as the thousandfold difference in the K_D for glutamine and the K_i for γ-glutamyl hydrazide (Weiner & Heppel, 1971) indicated.

We have found that substances that abolish $\Delta\bar{\mu}_{H^+}$ inhibit glutamine transport in isolated membrane vesicles. This was true in the presence of relatively high concentrations of the exogenous energy source (20 mM), as well as in vesicles that had been preincubated with the exogenous energy source. These observations suggest a role for $\Delta\bar{\mu}_{H^+}$ in glutamine transport in vesicles and agree with conclusions drawn previously by others (Singh & Bragg, 1977, 1979; Plate et al., 1974; Plate, 1979). However, the data presented here do not conclusively rule out the possibility that these inhibitors might act by preventing the uptake and proper metabolism of the exogenous energy source by vesicles. Further work is needed to unambiguously define the mechanism of action of compounds such as CCCP, cyanide, and azide on shock-sensitive transport.

The identity of the immediate energy donor for shock-sensitive transport in *E. coli* has not definitely been established. ATP (Berger, 1973; Berger & Heppel, 1974) and acetyl phosphate (Hong et al., 1979) have both been suggested to serve such a role. However, data presented here argue against a role for ATP or acetyl phosphate in the energization of glutamine transport in vesicles. Likewise, our data indicate that glutamine transport is not driven solely by $\Delta\bar{\mu}_{H^+}$. Clearly, some additional factor, either besides or in addition to ATP, acetyl phosphate, or $\Delta\bar{\mu}_{H^+}$, is required for glutamine transport in vesicles. Our results indicate that this unknown compound can be generated by vesicles from exogenous pyruvate, providing the vesicles contain incorporated NAD and are prepared

from strains containing active acetate kinase and phosphotransacetylase. This compound can also be generated from succinate, in a manner independent of acetate kinase, phosphotransacetylase, and NAD. Further work with the system described here should shed light onto the identity of this factor.

An interesting sidelight to these considerations is the considerable metabolic capabilities of the vesicle preparations described here. We find that vesicles are capable of incorporating ^{32}P from inorganic phosphate into ATP, ADP, AMP, GTP, acetyl phosphate, and several unidentified phosphate-containing compounds (Figure 5). These results indicate the presence of several enzymatic activities in vesicles. This in turn suggests that, although the concentration of cytoplasmic enzymes and cofactors is several orders of magnitude lower inside vesicles than in whole cells, the levels of these compounds are sufficient to impart on vesicles the ability to perform several biochemical transformations. The significance of this fact with respect to our work with glutamine transport cannot be understated, since without these capabilities, our vesicle preparations would probably be unable to perform pyruvate- or succinate-driven glutamine transport.

Finally, we have presented evidence suggesting that vesicles prepared from the glutamine binding protein containing parent of PSM116, PSM2, retain significant ability to take up glutamine, presumably because of the presence of large amounts of binding protein associated with the vesicles. Thus, the uptake seen is independent of added binding protein, is not dependent on added energy, and is not removable by various types of washing of vesicles or spheroplasts. The nature of the association of binding protein with membranes in PSM2 awaits further study.

Added in Proof

More recent work has shown that "clear" vesicles, prepared by diluting spheroplasts into a much larger volume of lysis buffer than described here, are impaired in their ability to use D-lactate, pyruvate, or succinate to drive glutamine transport. This result stresses even further the importance of metabolic capabilities of the vesicles described here and should accelerate the energetic study of glutamine transport in vesicles.

Registry No. L-Glutamine, 56-85-9; potassium, 7440-09-7; phosphate, 14265-44-2; pyruvic acid, 127-17-3; succinic acid, 110-15-6; D-lactic acid, 10326-41-7; γ-glutamyl hydrazide, 1820-73-1; *S*-carbamyl-L-cysteine, 2072-71-1; acetate kinase, 9027-42-3; phosphotransacetylase, 9029-91-8.

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