BINDING PROTEINS AND MEMBRANE TRANSPORT*

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Bacterial cells have developed two basic types of active transport systems. One of these transport systems is a membrane-bound system and can be observed in membrane vesicle preparations prepared by the methods described in the previous presentation by Kaback.[1] Dr. Kaback described the transport properties of membrane vesicles from gram-negative bacteria that retain the membrane-bound transport system. He has shown that these membranes retain all components of the transport system except an energy source, which is necessary for the system to produce active transport. The membrane-bound systems derive their energy from an "energy-rich membrane state," which can be formed in a variety of ways such as respiration, ATP hydrolysis, or proton gradient-forming conditions.[3, 4]

A second type of active transport system that I would like to describe is a system that requires a shockable binding protein to produce active transport.[57-61] It appears that the binding protein transport systems obtain their cellular energy more directly from phosphate bound energy derived from glycolysis or oxidative phosphorylation.[2] These low molecular weight binding proteins can be removed from the bacterial cells by a cold osmotic shock treatment. In general osmotic shock treatment selectively destroys the type-two transport systems for which binding proteins can be isolated and has little effect on type-one transport systems in which the receptor site is tightly bound to the membrane.

The membrane of *E. coli* is a complicated triple-layer structure and the binding proteins appear to occupy the space between the outer and inner membranes of the cell envelope. The mild treatment of cold osmotic shock was worked out by Neu and Heppel.[5] The procedure is illustrated in FIGURE 1. In the first step cells are harvested in middle or late log phase and washed several times with Tris-HCl, pH 7. The washed cells are suspended in 40 to 80 volumes of room temperature 20% sucrose solution containing 0.1 mM EDTA. The cell suspension is stirred gently for 10 minutes and then centrifuged. The pellet is rapidly resuspended in 40 to 80 volumes of ice-cold distilled water containing 0.1 mM $MgCl_2$. The suspension is stirred for 10 minutes and centrifuged. The supernatant fluid is called the "shock fluid" and contains a number of hydrolytic enzymes as well as the binding proteins for various solutes. Usually about 4% of the total cellular protein is lost from the cells during this treatment. The procedure must be modified for certain strains that are more sensitive to osmotic shock treatment such as *Pseudomonas* species.

When we treated *E. coli* K12 by the osmotic shock treatment we found

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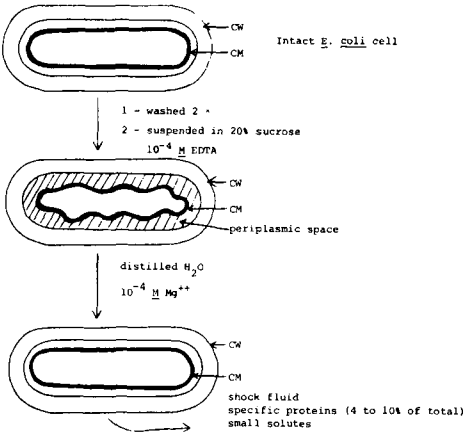


FIGURE 1. Schematic diagram of the process of osmotic shock and its effect on the bacterial cell.

that the transport activity for the branched-chain amino acids was greatly decreased. We examined the shock fluid and discovered leucine-binding activity by equilibrium dialysis.[6, 7]

Following concentration by ultrafiltration, the shock fluid was prepared for DEAE-cellulose by desalting on a Biogel-P10 column. The protein solution is applied to a DEAE-cellulose column equilibrated with 5 mM Tris-HCl, pH 7.6. The column was eluted with a linear gradient of sodium chloride. Three peaks with binding activity for branched-chain amino acids were found, corresponding to the LIV-binding protein, an isoleucine-preferring protein, and the leucine-specific protein.[8, 13] The elution pattern is illustrated in FIGURE 2. All three binding proteins crossreact with antibody prepared from the LIV-I binding protein, suggesting a considerable structural similarity. The bars in FIGURE 2 indicate crossreaction with anti-LIV-I antibody. The leucine-specific protein is purified to homogeneity by preparative polyacrylamide gel electrophoresis and crystallized from ammonium sulfate.[8] The isoleucine-preferring protein has not been extensively studied. The LIV-binding protein fraction from the DEAE-cellulose is purified with two steps, hydroxylapatite chromatography and isoelectric focusing. A final purification step can be achieved by

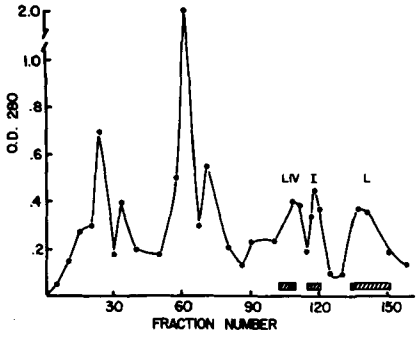


FIGURE 2. Elution profile of the DEAE-cellulose fractionation of the osmotic shock fluid. Crossreactive material is indicated by bars. (From Rahmanian *et al.*[13] By permission of the *Journal of Bacteriology*.)

crystallization from 50–55% α-methyl-2,4-pentanediol.[7] Two crystal forms exist that depend on the pH at which crystallization takes place. At neutral pH values, needle-like crystals have been obtained,[7] while at pH 4 hexagonal columns occur (FIGURE 3). Initial x-ray diffraction studies have shown good resolution and suggest the possibility of doing structural studies by this technique. A large number of other binding proteins for amino acids, ions, sugars, and vitamins have been isolated by similar techniques from bacteria. TABLE 1 lists some of these binding proteins and some physical properties that have been measured. References for the purification technique have also been included.

As indicated in this Table, the leucine-specific binding protein has a molecular weight of 37,000,[8] very similar to that obtained for the LIV-binding protein.[8] The LIV-binding protein is very stable to various denaturation procedures. The active binding form is the thermodynamically favored conformation, and denaturation by heat treatment, urea, or guanidine-HCl is fully reversible. This is a general feature of the periplasmic binding proteins. The K_d for leucine is about 10^{-6} M for both the branched-chain amino acid binding proteins. The similarity of the two proteins is confirmed by the crossreactivity either protein exhibits when antibodies are prepared to leucine-specific or LIV-protein alone. Crossreactivity is also shown between the arabinose- and the galactose-binding proteins of *E. coli* B/r.[9, 10]

Specific antibodies have been used to localize the binding proteins to the cell envelope.[11, 12] In the study of Nakane *et al.*,[12] antibodies to the LIV-protein

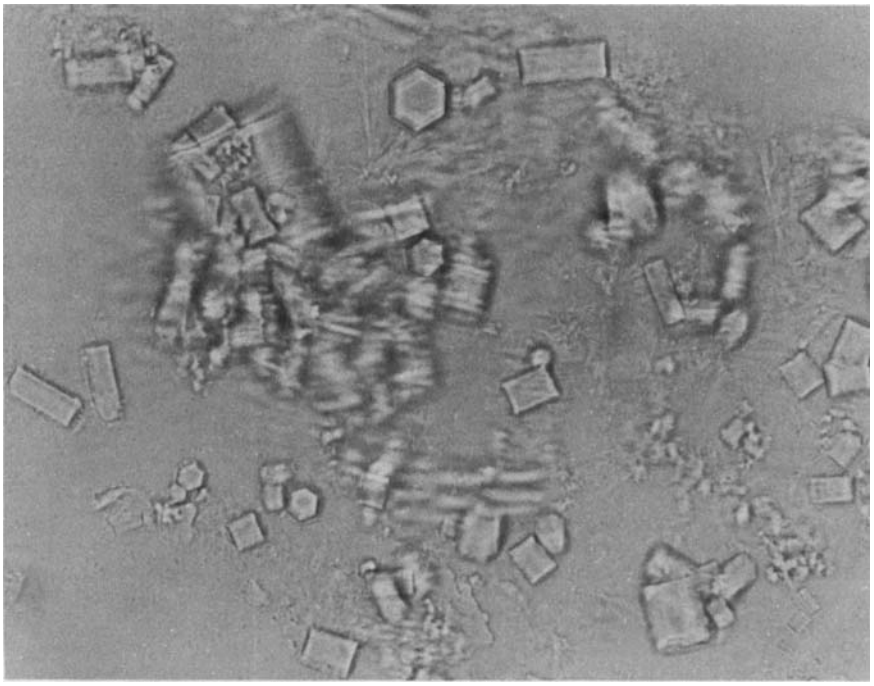


FIGURE 3. Crystals of the LIV-binding protein at pH 4.

TABLE 1
PHYSICAL PROPERTIES OF AMINO ACID-, SUGAR-, ION, AND VITAMIN-BINDING PROTEINS

Binding Protein	Organism	Molecular Weight	Cysteine Content	Dissociation Constant (10^{-6}M)	Specificity	References
Amino Acids						
Lysine, Arginine, Ornithine	*E. coli* K12	28,000	2	3, 1.5, 5	Specific	37
Arginine	*E. coli* K12	27,700	2	0.03	Specific	38
Histidine	*S. typhimurium* LT2	25,500	2	0.15	His > Arg > Lys > citrulline > azoserine	39
Cystine	*E. coli* W	27,000	3	0.01	Cystine > diaminopimelate	40
Glutamine	*E. coli* K12	27,000	0	0.3	Specific	41
Glutamate and Aspartate	*E. coli* K12	31,000	2	0.7, 1.2	Specific	42
Leucine, Isoleucine, Valine	*E. coli* K12	36,000	1	1	Leu = Ile > Val > Thr > Ala	7, 8
Leucine	*E. coli* 7	37,000	–	0.7	Leu > Trifluoroleucine	8, 13
Phenylalanine	*Comamonas*	–	–	0.2	–	43
Tryptophan	*Neurspora crassa*	–	–	80	Phe > Trp > Leu	44
Sugars						
Arabinose	*E. coli* B/r	39,000	2	0.3	Ara = Gal ≫ Glu	9
Galactose	*E. coli* K12	36,000	0	0.5 and 10	Gal = Glu > Ara	32, 45, 46
Ribose	*S. typhimurium* LT2	30,400	0	0.33	Specific	47
Maltose	*E. coli* K12	40,500	–	1.5 and 10	Maltose > several maltodextrins	48
Ions						
Calcium	Chick, intestinal mucosa	24,700	3	26	Calcium > strontium > barium	49, 50
Phosphate	*E. coli* K10	42,000	–	0.8	–	51
Sulfate	*S. typhimurium* LT2	32,000	0	20	Sulfate > chromate	52
Vitamins						
Thiamine	*E. coli* W	–	–	0.03	Thiamine > thiamine monophosphate > thiamine diphosphate	53, 54
Cyanocobalamine	*E. coli* B	22,000	–	0.006	–	55

were used in an enzyme-labeled technique. Late log phase cells were fixed in acetone, washed with phosphate buffer, and treated with rabbit antibody to the binding protein. The cells were then washed and allowed to react with sheep anti-rabbit globulin that had been conjugated to horseradish peroxidase. The fixed cells were stained cytochemically for peroxidase. The location of the insoluble product, which reacts with osmium tetraoxide, could be observed in the electron microscope. Using unimmunized rabbit serum as control, it was possible to localize the reaction to the cell wall or cell membrane. Using the fluorescent reagent, diazo-7-amino-1,3-naphthalene disulfonate, Pardee and Watanabe[11] obtained evidence that the sulfate-binding protein was located internal to the cell wall, but external to the plasma membrane. This reagent penetrates the cell wall but not the cellular membrane.

Multiplicity of Branched-Chain Amino Acid Systems

When the kinetics of leucine transport were examined over a wide concentration range the reciprocal plots of the initial rates of uptake were distinctly biphasic, indicating heterogeneity in leucine transport.[13] The multiplicity of branched-chain amino acid systems has been confirmed in other laboratories.[8, 13-15, 56] We have used a combination of kinetic, genetic, and biochemical studies to describe the number of transport systems that serve for leucine, isoleucine, and valine.

High-Affinity Uptake Systems

The high-affinity component of the kinetic plot is composed of at least three transport systems. The major high-affinity system is called the LIV-I system. This system has a broad specificity since it serves for leucine, isoleucine, valine, threonine, alanine, homoserine,[16] cysteine, serine, methionine, and a variety of analogs.[16, 17] Additional minor high-affinity systems are represented by a leucine-specific system (L-S system)[8, 13] and an isoleucine-preferring system (I-system).[18] These three systems each have approximately the same Km value for uptake of their preferred substrate (0.2 μM). Thus, they appear collectively as one component in the kinetic plots of uptake of the respective substrate. All three of these systems are repressible by leucine in the growth media and are sensitive to osmotic shock treatment. The three proteins (isolated as described and shown in FIGURE 2) have specificities that correspond to the three high-affinity transport systems described above. In wild type *E. coli* the LIV-I system constitutes about 75 to 85% of the high-affinity uptake of leucine and isoleucine, while the specific uptake systems constitute the balance of the high-affinity transport of these amino acids.

Low-Affinity Transport of Leucine, Isoleucine, and Valine

The low-affinity component of the kinetic plot of leucine uptake is referred to as the LIV-II transport system. This component is not sensitive to osmotic shock treatment and can be observed in shocked cells. It is not subject to repression by growth of the cells on leucine. Kinetic plots of leucine uptake

in shocked cells or cells grown in the presence of leucine are essentially linear and provide a good measure of the LIV-II transport component. The Km values for leucine and isoleucine are from 2 to 4 micromolar.[13] The Km value for valine is around 10 to 20 micromolar.

In contrast to the LIV-I system, the LIV-II transport system is much more specific for leucine, isoleucine, and valine. Some analogs of the branched-chain amino acids such as norleucine[50] have low affinity for the LIV-II system. Since the LIV-II system is not sensitive to osmotic shock treatment, it can also be observed in membrane vesicle preparations of bacteria as described in the previous presentation by Kaback. In wild-type *E. coli* K12 the LIV-II system ordinarily constitutes about 10 to 15% of the uptake of 1×10^{-5} M leucine. At this concentration of leucine both high- and low-affinity transport systems would be saturated.

Regulation of Leucine Transport

We have previously shown that when cells are grown in nutrient medium or in media containing leucine the transport activity and binding activity is repressed.[7] The represson by leucine of both transport and shockable-binding activity is coordinate, supporting the view that the binding protein is involved in the rate-limiting step in the high-affinity leucine transport system (LIV-I).

Since the biosynthesis of leucine is also repressed by external leucine, we examined the possibility that biosynthesis and transport of leucine shared common regulatory components. We examined the transport activity in mutant strains with derepressed leucine biosynthetic enzymes obtained from Dr. Umbarger. We found that the transport was normal and still regulated by leucine in the growth media. In addition a mutant with derepressed leucine transport activity, strain E0312, showed normal regulation of the biosynthetic enzymes for leucine.[19] These data are summarized in TABLE 2.

TABLE 2

THE SEPARATION OF BRANCHED-CHAIN AMINO ACID TRANSPORT AND BIOSYNTHESIS

Strain	Phenotype	Percent of Parent	
		Biosynthetic Enzymes	LIV Transport System
CU5	wildtype	100	100
CU5001	derepressed *leuABCD, ilvB*	700*†	100†
CU5002	derepressed *leuABCD, ilvB, ilvADE*	439*†	119†
EO303	wildtype	100	100
EO312	derepressed branched-chain amino acid transport and binding proteins	79†	200‡

* Data are for the *ilvB* gene product, acetohydroxy acid synthetase. Similar results were obtained for the *leuB* gene product, isopropyl malate dehydrogenase.

† Data from Reference 19.

‡ Data from Reference 13.

Transport Mutants

Many of the mutant selection procedures are designed to use amino acid auxotrophs. The high-affinity transport systems that require binding proteins are greatly reduced in leucine auxotrophs since growth on leucine leads to their repression.

In order to obtain mutants that were no longer repressed by the addition of leucine, we plated an auxotroph on D-leucine. D-Leucine is not transported efficiently enough to provide for a source of L-leucine via racemization in an auxotroph unless a derepression in transport occurs. When a wild-type *E. coli* K12 strain that has a lesion in leucine biosynthesis is plated on 200 mg/L D-leucine, D-leucine-utilizing mutants (DLU) were obtained. Most of these mutants had normal transport; however, about 3% of these mutants had greatly increased D- and L-leucine and isoleucine transport activity.[20, 21] These mutants showed corresponding increases in the levels of the various binding proteins for leucine, isoleucine, and valine.

One class of these mutants, represented by strain E0312,[18] shows derepressed levels of the high-affinity transport systems, and the addition of leucine to the growth media no longer represses the level of the high-affinity transport and the level of the three binding proteins. The kinetics of the high-affinity transport of leucine shows a two- to threefold increase in maximal capacity without significant changes in the Km value. This mutation has been partially mapped by genetic techniques and appears to be about 2% linked to the *pyrD* locus at minute 21 of the *E. coli* chromosome.[22]

A second class of D-leucine utilizing mutants is represented by strain E0318. This mutant, like the previous mutant, also has a two- to threefold increase in leucine transport activity; however, the major increase in leucine transport results from an increased level of the leucine-specific system. The leucine-specific system makes up about 50% of the leucine uptake in this strain. The leucine-specific binding protein is also increased severalfold.[13] The LIV-I transport system appears unchanged in the E0318 strain and is still repressed by leucine. This regulatory mutation has been mapped and is 60 to 70% linked to the *aroA* locus at minute 20 on the *E. coli* chromosome.[22] These regulatory mutants show increased transport activity as a result of the increased binding proteins, again indicating that the binding proteins are involved in the rate-limiting step in high-affinity branched-chain amino acid transport systems.

Defective Transport Mutants

The D-leucine-utilizing strains E0311 and E0318 are able to utilize D-leucine because of increased transport activity for D-leucine. Using these strains, we applied a penicillin selection in the presence of D-leucine to isolate transport mutants among the survivors. Several mutants were isolated that showed greatly decreased D- and L-leucine transport activity.[13] The transport defect in these mutants was shown to be in the high-affinity transport systems and the LIV-II low-affinity membrane-bound system was relatively unaffected. We observed three types of transport mutants in these studies. One type proved to be defective in a large number of transport systems and was therefore considered to have a general membrane defect. The parental DLU strain E0311, which

had the high-affinity systems derepressed, was used to obtain a second type of mutant. This mutant, strain E0323, showed a loss in all the high-affinity systems, but appeared to have normal binding protein levels. Strain E0323 apparently has a defect in a common component for the binding protein transport systems. A third type of transport mutant was derived from the parental DLU strain E0318, which has high leucine-specific activity. This mutant strain, E0321, has lost only the specific leucine transport system. When we examined the shock fluid for leucine-specific binding activity, none was found. A protein fraction corresponding to the leucine-specific protein was eluted from DEAE columns at a slightly slower rate than authentic leucine-specific binding protein. This protein fraction could be detected in the eluate by its crossreactivity with antibody to the LIV-I binding protein. This strain, containing a mutation in the structural gene of the leucine-specific binding protein, is being mapped to establish the position of the structural gene on the *E. coli* chromosome.

Analog-Resistant Mutants

The use of analogs of leucine such as azaleucine and trifluoroleucine have not been effective in obtaining mutants with defective binding proteins. The inability to obtain such mutants may have several causes. First, these analogs are not very toxic to wild-type strains. Second, they are ineffective for selection methods using leucine auxotrophs because the requirement for exogenous leucine in these strains prevents uptake of the analogs. In addition many strains are able to develop resistance to moderate levels of the analog by derepressing the leucine biosynthetic pathway. The DLU mutant strains with increased leucine uptake described above are much more sensitive to low levels of azaleucine or trifluoroleucine.

An examination of azaleucine transport activity in these strains shows that it can enter the cell by the LIV-I system and by the general aromatic amino acid transport system called *aroP*.[17] Although the derepressed transport system can increase the sensitivity of a cell to azaleucine, extensive studies have indicated that a variety of cellular processes can alter the azaleucine toxicity.[17]

A large number of azaleucine-resistant mutants have been examined for leucine transport activity. Approximately 50% of the mutants have defective leucine transport. Some of these mutants have membrane defects and are thus pleiotropic transport mutants. The rest of the transport mutants have a specific defect in the high-affinity leucine transport system. An examination of several hundred has failed to yield a binding-protein negative mutant; therefore, we conclude that the predominant transport mutant that derives from the analog resistance selection is defective in a common component of the high affinity systems other than the binding protein component. These mutants will be useful for characterizing the nonbinding protein components of the high-affinity leucine transport system.

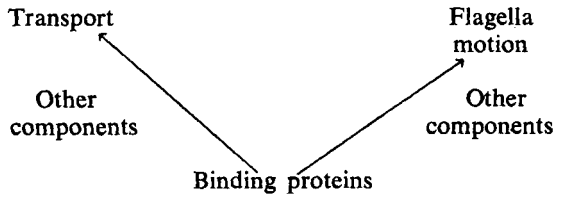
Valine-Resistant Mutants

E. coli K12 is extremely sensitive to growth inhibition by low levels of L-valine since valine causes feedback inhibition of acetohydroxy acid synthetase,

a common enzyme in valine and isoleucine biosynthesis. Valine-induced growth inhibition is relieved by the addition of isoleucine to the medium. The most common class of valine-resistant mutants obtained is the regulatory mutant in the biosynthetic pathway for isoleucine and valine. Various mutations leading to the valine-resistant phenotype have been mapped at six different chromosomal loci in *E. coli*.[23] Guardiola and Iaccarino have used valine resistance in the presence of leucine and methionine to obtain several types of mutants.[15, 24] In addition to finding regulatory mutants for the *ilv* pathway they identified transport mutants. Some of these mutants have been genetically mapped, but the biochemical characterization is not sufficient at the present time to assign the defect to a specific branched-chain amino acid binding protein.

Requirement of Binding Proteins in Chemotaxis

Chemotaxis by *E. coli* has been the subject to extensive studies[25-29] and will not be covered now except to indicate the relationship of certain binding proteins to chemotaxis. Although not all chemo receptors are shockable binding proteins, a number of the sugar-binding proteins appear to be absolutely required for chemotaxis toward the respective sugar. Chemotaxis of *E. coli* toward galactose,[26, 27] ribose,[28] and maltose[29] requires the presence of the specific sugar-binding protein. There are additional components required for both transport and chemotaxis even though the binding protein serves as a common element. The relationship of the binding proteins to transport and chemotaxis can be diagrammed as follows:



There are transport-specific components not required for chemotaxis and chemotaxis-specific components not required for transport as illustrated in this diagram. These additional components have been identified by genetic techniques.[25] Galactose transport mutants have been obtained that show normal chemotaxis for galactose and certain chemotactic mutants have normal transport. These latter types of mutants both have normal levels of galactose-binding protein.

Role of Binding Proteins in Transport

A variety of indirect evidence has accumulated to support a direct role of the binding protein in solute transport by the high-affinity shock-sensitive transport systems. These lines of evidence include: (a) osmotic shock treatment causing a parallel loss in transport activity and binding protein; (b) similarity of the kinetic constants; (c) parallel regulation of transport and binding protein activities; (d) localization of binding proteins to the periplasmic space; and (e) reversion of transport negative mutants that do not contain functional

binding proteins, which always results in a recovery of both transport and binding protein activities.

Reconstitution studies to find direct evidence for a role of binding proteins have been generally discouraging, although genetic studies have provided direct evidence for a role of certain binding proteins.

Mutants with Altered Binding Proteins

Starting with strains that can utilize D-histidine, Ames and Lever were able to isolate mutants with the histidine-binding protein (J) missing.[30, 31] Revertants of these histidine transport mutants (induced by the frameshift mutagen ICR191 in a *hisJ* mutant that was itself induced with ICR191) were obtained that contained *hisJ* proteins with altered chemical and physical properties. The revertant strain was more temperature-sensitive for both growth and histidine transport activity than was the parental strain. The histidine-binding protein isolated from the temperature-sensitive revertant was also more sensitive to heat treatment than normal histidine-binding protein. These data provide direct evidence for a role of the histidine-binding protein in the high-affinity histidine transport system.

Direct evidence that the galactose-binding protein plays a role in β-methyl-D-galactoside transport was provided by obtaining mutants of *E. coli* that contained altered galactose-binding proteins that resulted in an altered chemotaxis[26] and an altered transport of galactose.[32] The amino acid structure of the galactose-binding protein was found to be altered leading to a lower affinity for galactose which was reflected simultaneously in a higher Km value of transport and a higher threshold for the detection of positive chemotaxis specifically for galactose.[33]

As indicated in an earlier section we have also isolated a mutant that appears to be in the structural gene of the leucine-specific binding protein. This mutation produces a CRM protein that results in the complete loss of leucine-specific transport activity.

Recently Kustu and Ames[34] obtained a histidine transport mutant from *Salmonella typhimurium* that had lost the high-affinity histidine transport, although it retained normal levels of the histidine-binding protein. This mutation proved to be in the structural gene of the histidine-binding protein, suggesting that a site on the binding protein other than the recognition site may serve for binding additional membrane components.

Extensive genetic studies[35, 36] on galactose transport have shown that mutants of the β-methyl-D-galactoside transport system can all be placed in one of three classes using genetic complementation analysis.[35] The three genes have been called *mglA*, *mglB*, and *mglC* and are genetically linked to the *his* loci of the *E. coli* chromosome. The *mglB* was determined to be the structural gene for the galactose-binding protein, and the products corresponding to *mglA* and *mglC* appear to be additional membrane-bound components of the galactose transport system. Robbins and Rotman[36] found that the galactose-binding protein was required for active transport of galactose, although *mglB* mutants could grow on high levels of galactose. They concluded that the binding protein was not involved in the translocation step in transport.

FIGURE 4 presents a diagram of the possible role of the binding protein in galactose transport. Components A and C are believed to be membrane

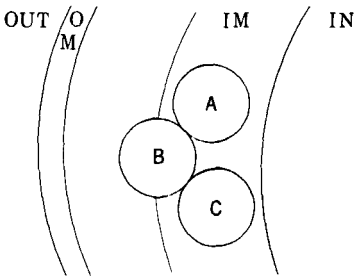


FIGURE 4. The role of the galactose-binding protein in galactose transport. OUT, outside; OM, outer membrane; IM, inner membrane; IN, inside of cell.

components of the translocation process and the binding protein (B) creates an active transport system by increasing the affinity of the membrane-bound systems for galactose. Robbins and Rotman[36] suggest that the affinity of the system may be as much as 1000 times greater in the presence of the binding protein, although the capacity of the system is not altered by the presence of the binding protein.

Silhavy *et al.*[32] have prepared an extensive review of the evidence for the role of the galactose-binding protein. In agreement with Robbins and Rotman,[36] they do not believe that the binding protein for galactose is involved in the translocation step. They review the evidence for two sugar binding sites on the galactose-binding protein. These authors suggest that one site is the recognition site for galactose and that the other is a glycoprotein recognition site. Their model predicts that either or both of components A and C in the membrane may be a glycoprotein, and they have experiments under way to test this possibility.

The results from our laboratory on the role of the leucine-binding protein in transport are not in disagreement with those found for galactose. At the present time, most of our data suggest that LIV-I and LIV-II are parallel systems and not connected by a series of steps, although some mutants appear to be defective in both LIV-I and LIV-II transport systems, which suggests they may share a common component.

Conclusions

The recent studies have clearly established two types of active transport systems. One type is membrane-bound and can be observed in membrane vesicles and the other type is osmotic-shock-sensitive and requires binding proteins to produce active transport. It appears that the membrane-bound systems derive cellular energy from an energy-rich membrane state which can be formed from respiration or ATP-hydrolysis, while the binding protein systems are more directly coupled to phosphate bond energy derived from glycolysis or oxidative phosphorylation.

The following conclusions concerning the role of the binding proteins are offered:

1. The binding proteins are present in relatively large amounts ($\sim 10^{-6}$ or 10^{-5} M) and appear to reside in the periplasmic space.

2. They do not appear to be involved in solute translocation steps, although they contain a second binding site that could interact with membrane components.

3. The binding proteins appear to increase the affinity of the transport system for the solute by interacting with a membrane component. This may result from the possibility that the solute-binding protein complex is the actual substrate for the membrane transport system.

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Discussion

DR. TOSTESON: I wonder whether binding proteins function so poorly as transporters because their affinities for the transported substances are too

high and the rate coefficient for release is too low? To that end it would be interesting to know whether there is any evidence either in bacterial systems or in vesicles that binding proteins can actually go all the way through the bilayer.

DR. OXENDER: The binding proteins don't appear to have measurable affinities; also we've sent protein to Thompson at Virginia, and he has attempted to see whether they would have any affinity for lipid bilayers, and his experimental results were negative. We've also taken liposomes and loaded them with radioactive leucine and then added the binding protein to see if binding proteins will stimulate the efflux of leucine; again the results were negative.