GLUTAMATE-BINDING PROTEIN AND ITS RELATION TO GLUTAMATE TRANSPORT IN ESCHERICHIA COLI K-12

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SUMMARY

A highly specific, energy-dependent active transport system of glutamate has been found in Escherichia coli K-12. Mutants capable of utilizing glutamate as the sole carbon and energy source exhibit several-fold higher rates of glutamate uptake than the wild-type parent. Spheroplasts prepared from one such mutant, SC7, retain only 10-30% of the glutamate uptake capacity of intact cells. The capacity of spheroplasts for glutamate uptake can be fully restored by the addition of concentrated supernatant fluid obtained in the preparation of spheroplasts. This supernatant contains a protein fraction capable of binding L-glutamate with a K_m of 6.7 x 10^{-6}M. L-glutamate-γ-methyl ester, a competitive inhibitor of glutamate uptake also inhibits competitively the binding of glutamate to the protein. L-alanine, a non-competitive inhibitor of glutamate uptake, inhibits its binding by the protein in a non-competitive fashion.

Recent studies have shown that proteins located in the "periplast" region of gram-negative bacteria are selectively released during the preparation of spheroplasts from these bacteria, or even upon mild shock treatment (1). Among these proteins, which amount to 5-10% of total cell protein, several have been described which are capable of specifically binding certain low-molecular-weight compounds. Thus, proteins specifically binding sulfate (2,3) and phosphate (4), galactose-(5,6) and arabinose-(7,8) binding proteins, and proteins specific for certain amino acids, such as leucine (9,10), histidine (11), arginine (12) and phenylalanine (13), have been described. Each of these binding proteins is believed to play an essential role in the functioning of the respective transport system, specific towards a given permeant or group of related permeants. Cumulative evidence from different bacterial transport systems indeed seems to indicate the involvement of binding proteins in the transport process. However, as yet, very little is known on the genetics of the binding proteins and there is hardly any information at all on their

actual role in transport and on the genetic and metabolic control of their synthesis and/or activity.

Our laboratory has been engaged for some time in studies of glutamate transport in E. coli K-12. We have shown (14,15) that glutamate is taken up via a highly specific temperature- and energy-dependent active transport system. L-glutamate uptake is competitively inhibited by structural analogs of the permeant (e.g. L-glutamic acid-γ-methyl ester) and non-competitively by compounds metabolically related to glutamate (e.g. L-alanine, α-ketoglutarate). Three genes which determine and control glutamate transport have been defined by mutant studies and mapped: a structural gene, gltS (mutations resulting in loss of ability to utilize glutamate as sole carbon source and in a sharp decrease in affinity of the uptake system for glutamate); a control gene, gltC, closely linked to gltS (mutations affecting Vmax of uptake, but not Km); and a regulatory gene, gltR, unlinked to the former two, making a cytoplasmic repressor-like product (temperature-sensitive mutants capable of utilizing glutamate when grown at 42°C, but not at 30°C). Mutations at the gltC locus resulting in a several-fold increase in the rate of glutamate entry into the cell do not affect its rate of exit from cells preloaded with the radioactive amino acid (16).

The subject of this communication is a glutamate-binding protein fraction obtained from E. coli K-12 CS7, a glutamate utilizing K-12 mutant. Several lines of evidence are presented which strongly indicate that the glutamate-binding protein is a component of the glutamate transport system.

MATERIALS AND METHODS

Bacterial strain. Escherichia coli K-12 CS7, a methionine auxotroph capable of utilizing glutamate as the sole source of carbon was used throughout this work.
Growth medium. The basal medium of Davis and Mingioli (17) from which citrate was omitted, supplemented with DL-methionine, 50 µg/ml and sodium succinate, 1% (unless otherwise specified), was used.
Growth of bacteria. Cultures were grown with aeration at 37°C. For uptake experiments the bacteria were harvested in mid-logarithmic phase. For the preparation of glutamate-binding protein early stationary phase cultures were used.
Preparation of spheroplasts. Spheroplasts were prepared according to Repaske (18), except that the concentrations of several reagents were changed to the following: sucrose, 25%, lysozyme, 0.1 mg/ml, bacteria, 5.5 x 10^{10} cells/ml; 2-mercapto-ethanol, 0.001 M, was added. The mixture was gently stirred at 20°C for 6 minutes.
Preparation of crude glutamate-binding fraction. Early stationary phase cells were treated as above. The spheroplasts were removed by centrifugation in the

cold at 15,000 x g for 20 minutes. The supernatant was dialyzed against 20 volumes of potassium phosphate, pH 7.0, 0.005 M, with 2-mercaptoethanol, 0.001 M (A buffer) for 18 hrs at 4°C, with three changes of buffer, and lyophilized. The residue was taken up in a small volume of potassium phosphate, pH 7.0, 0.05 M, with 2-mercaptoethanol, 0.001 M (B buffer) and dialyzed against 50 volumes of B buffer with 0.5% chloroform for 12 hrs at 4°C, with two changes of buffer.

Glutamate binding. Glutamate binding was determined by equilibrium dialysis. A dialysis sac with 0.5 ml of B buffer (control) and one with 0.5 ml of the crude glutamate-binding fraction were placed in test-tubes containing 4 ml of B buffer with 0.5% chloroform and L-^{14}C-glutamate (specific radioactivity of 11 mcurie/mmole; The Radiochemical Centre, Amersham, Bucks., England), 2 x 10^{-6} M (except where otherwise stated). The tubes were shaken at 4°C for 22 hrs and 0.05 ml aliquots of the external medium and of the dialysis sac contents were applied to filter discs, dried at 100°C for 30 minutes, placed in 10 ml scintillation liquid and counted as previously described (19).

Analysis of bound radioactivity. After equilibrium dialysis aliquots of external medium and of the dialysis sac contents were heated at 95°C for 15 minuts and applied to Whatman 3MM paper. Chromatograms were developed in water-saturated phenol with 0.3% ammonia and traces of KCN. The paper was dried and cut into 1.5 x 5 cm strips and radioactivity determined by liquid scintillation as above.

Restoration of glutamate uptake capacity of spheroplasts. Spheroplasts, prepared as above, were diluted in 100 volumes of Tris-HCl buffer, pH 8.0, 0.002 M, containing sucrose, 25%, $MgSO_4$, 0.02 M, sodium succinate, 1% and chloramphenicol, 200µg/ml (C buffer). The material tested for its ability to restore glutamate uptake was added and the mixture was incubated with gentle shaking at 25°C for 15 minutes. L-^{14}C-glutamate (1 mcurie/mmole) was added to a final concentration of 5 x 10^{-5} M and 1 ml samples were then taken at frequent intervals into 4 ml of C buffer kept at 25°C and quickly filtered on Millipore filters (0.45 µ). The filters were dried and counted as above.

Enzyme assays. Glutamate dehydrogenase (GDH) and glutamate-oxaloacetate transaminase (GOT) activities of crude preparations and purified glutamate-binding fractions were measured as previously described (19).

RESULTS

Loss and restoration of glutamate uptake. Bacteria were grown in minimal medium with glycerol, 0.5%. One part of the culture was harvested in mid-log phase, suspended in C buffer and tested directly for its capacity for glutamate uptake (untreated control). The remaining culture was treated according to Repaske (18)

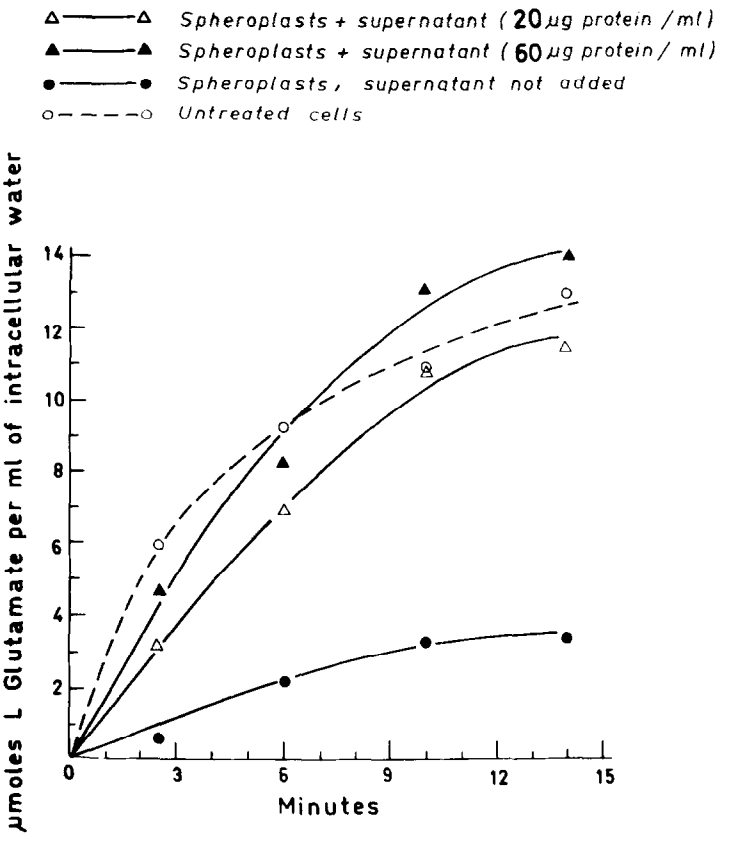

Fig. 1: Loss and restoration of glutamate uptake in spheroplasts. For experimental details see Materials and Methods.

as described above. After 6 minutes the spheroplasts were diluted in 100 volumes of C buffer and tested for glutamate uptake in the presence and in the absence of the crude glutamate-binding fraction. As shown in Fig. 1, the rate of glutamate uptake by unsupplemented spheroplasts was about 1/4 of that of untreated cells. (In some experiments the loss of activity in spheroplasts was even more pronounced). Addition of the crude glutamate-binding fraction (60 µg protein/ml) completely restored the capacity of spheroplasts for glutamate uptake.

Glutamate binding. Binding of glutamate was measured by equilibrium dialysis and the bound radioactivity was released by boiling and identified by paper chromatography as described in Materials and Methods. As shown in Table I, the crude supernatant preparation obtained after treating the cells with lysozyme according to Repaske (18), contained a non-dialyzable fraction with a high capacity for binding L-glutamate. Practically all of the glutamate bound was recovered as such after boiling.

Table I: Binding of L-^{14}C-glutamate by crude supernatant fraction.

L-^{14}C-glutamate concentration	L-glutamate bound		Radioactivity recovered as glutamate %	
M	nmoles/mg protein	Excess binding, %	Control	Protein-bound
2×10^{-6}	0.13	25.6	95.3	95.9
2×10^{-5}	0.51	9.8	96.0	96.6

A crude supernatant fraction containing 3.8 mg protein per ml, determined according to Lowry et al., (20), was used. Lysozyme and bovine serum albumin (10 mg/ml) controls showed no binding. For other details see Materials and Methods.

Ammonium sulfate fractionation. The crude preparation was centrifuged at 100,000 x g for 1 hr and fractionated with ammonium sulfate at 2°C. The precipitated fractions were dissolved in B buffer, dialyzed against 100 volumes of B buffer at 4°C for 12 hrs and tested for glutamate binding and for the activities of

Table II: Binding activity profile of crude supernatant fractionated with ammonium sulfate.

Fraction	Total protein	Binding activity		Enzyme activity	
	mg	nmoles glutamate per mg protein	Total %	nmoles/mg protein/min GDH	GOT
Crude	180.0	0.28	100.0	9.85	13.53
100,000g supernatant	146.0	0.31	89.8	1.17	8.35
Ppt. at 20% $(NH_4)_2SO_4$ saturation	1.8	0.42	1.5	nt*	15.67
" " 40% " "	15.7	0.04	1.2	nt	6.51
" " 60% " "	7.7	0.06	0.9	nt	2.84
" " 80% " "	63.7	0.14	1.8	nt	< 0.17
" " 90% " "	24.0	0.76	36.1	nt	< 0.17
" " 100% " "	6.5	1.17	15.2	nt	< 0.17

Solid ammonium sulfate ground to a fine powder was added slowly with constant mixing. The precipitates were dissolved and dialyzed overnight against 100 volumes of B buffer with two changes of buffer. For other details see Materials and Methods.

*nt - not tested.

glutamate dehydrogenase and glutamate-oxaloacetate transaminase. The results are summarized in Table II. More than 90% of the binding activity recovered was found in the fractions precipitated at 90 and 100% saturation with ammonium sulfate. These fractions were practically devoid of any GDH and GOT activity. Overall recovery of glutamate binding activity in the ammonium sulfate precipitates was about 64%, while the recovery of protein was ca. 82%. The precipitate and supernatant obtained at 60% saturation with ammonium sulfate were also tested for their ability to restore glutamate uptake by spheroplasts; only the supernatant was active (results not shown in Table).

Effect of pronase on glutamate binding by crude supernatant. Table III shows that the capacity of a crude preparation for binding glutamate was severely reduced by brief incubation with pronase at 37°C.

Table III: Effect of pronase on the glutamate binding capacity of supernatant partially purified by precipitation with ammonium sulfate.

Incubation time min	No pronase added		With pronase, 1 mg/ml	
	L-glutamate bound nmoles/mg protein	Relative activity, %	L-glutamate bound nmoles/mg protein	Relative activity, %
10	0.14	100.0	0.05	35.7
20	0.14	100.0	0.04	28.6
40	0.13	92.9	0.03	21.3

The supernatant fraction after treatment of crude glutamate-binding preparation with 76% saturated ammonium sulfate was used at a protein concentration of 5.2 mg/ml. The tubes with and without pronase were incubated at 37°C for the indicated length of time and then tested for glutamate binding as described in Materials and Methods.

Effect of transport inhibitors on binding. As one can see in Fig. 2, δ-methyl glutamate, a competitive inhibitor of glutamate transport (14) also inhibits competitively glutamate binding in vitro. L-alanine, which is a non-competitive inhibitor of glutamate uptake acts also as a non-competitive inhibitor of the binding reaction. It is noteworthy that the K_m of glutamate as well as the K_i values of δ-methyl glutamate and alanine as measured for the binding reaction, are lower than the corrosponding values for transport.

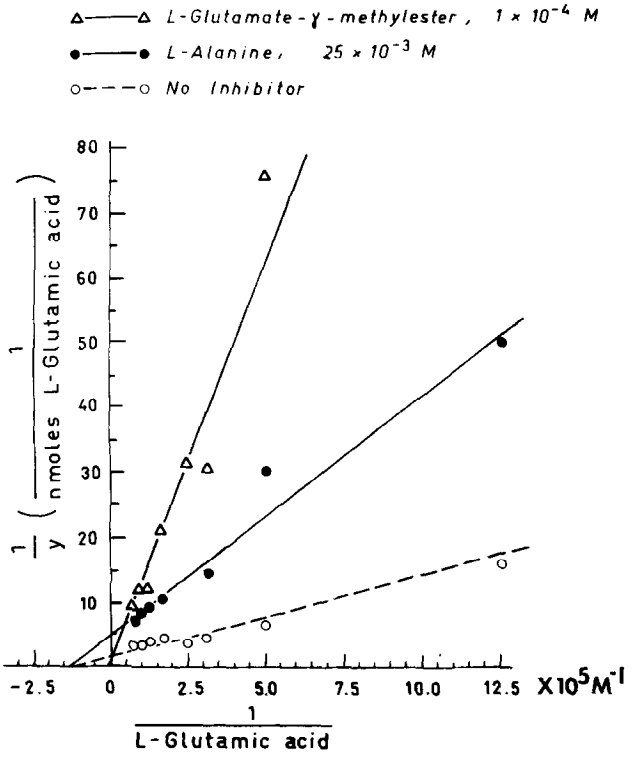

Fig. 2: Substrate saturation curve of glutamate binding and its inhibition by inhibitors of glutamate transport. Crude supernatant was used at a final concentration of 10 mg/ml. The K_m for L-glutamate calculated from the data in this Figure is 6.7 x 10-6 M, the K_i for L-glutamate-γ-methyl ester is 1.8 x 10^{-5} M and the K_i for L-alanine, 1.1 x 10^{-2} M. For other details see Materials and Methods.

DISCUSSION

The glutamate-binding component of E. coli, similarly to other bacterial binding proteins recently dicovered, is readily released into the medium by mild treatments which still enable the cell to retain over 90% of its proteins. It is therefore reasonable to assume that the glutamate-binding capacity resides in the outer membrane region. The precipitation of the glutamate-binding component by ammonium sulfate closely resembles that of the phosphate-, galactose- and arabinose-binding proteins described in the literature (4,6,7). That the glutamate-binding component is of protein nature is also strongly suggested by its inactivation by pronase. Our data indicate quite emphatically that this protein is an essential component of the glutamate transport system. Our argument is based on the following findings: a. Treatments which result in the release of binding protein from the cell bring about a sharp decrease in the cell's capacity for glutamate uptake. b. Full restoration of the transport capacity of treated cells

ensues upon the addition of partially purified binding protein. c. Binding activity and capacity for restoration of transport do not separate upon fractionation with ammonium sulfate. d. There is a close similarity in the mode of inhibition of glutamate uptake and binding by different compounds. The differences in the K_m and K_i values observed may be due to the large difference in the temperatures at which the two reactions were studied and/or to conformational changes in the protein as a result of its attachment to the membrane.

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