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CHARACTERIZATION OF GLUTAMATE TRANSPORT SYSTEM IN HYDROPHOBIC PROTEIN (H PROTEIN) OF *BACILLUS SUBTILIS*

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Summary

Hydrophobic protein (H protein) was isolated from membrane fractions of *Bacillus subtilis* and constituted into artificial membrane vesicles with lipid of *B. substilis*. Glutamate was accumulated into the vesicle when a Na^+ gradient across the membrane was imposed. The maximum effect of Na^+ on the transport was achieved at a concentration of about 40 mM, while the apparent K_m for Na^+ was approximately 8 mM. On the other hand, K_m for glutamate in the presence of 50 mM Na^+ was about 8 μM. Increasing the concentration of Na^+ resulted in a decrease in K_m for glutamate, maximum velocity was not affected. The transport was sensitive to monensin (Na^+ ionophore).

Glutamate was also accumulated when pH gradient (interior alkaline) across the membrane was imposed or a membrane potential was induced with K^+-diffusion potential. The pH gradient-driven glutamate transport was sensitive to carbonylcyanide *m*-chlorophenylhydrazone and the apparent K_m for glutamate was approximately 25 μM.

These results indicate that two kinds of glutamate transport system were present in H protein: one is Na^+ dependent and the other is H^+ dependent.

Introduction

Recently, we reported on the isolation of hydrophobic protein (H protein) which was found in the membrane fractions of *Bacillus subtilis* [1]. H protein contains several membrane protein components including amino acid carrier [2]. During the biochemical studies on H protein, we have found that H protein contained glutamate transport carrier and that the transport was dependent on both Na^+ and pH gradient across the membranes.

We report here our findings on the characteristics of glutamate transport system in H proteins of *B. subtilis.*

Materials and Methods

Isolation of H protein and formation of the vesicles. H protein was prepared as previously described [2]. Lipids of Bacillus (consist mainly of phosphatidylglycerol (60%) and phosphatidylethanolamine (25%)) were extracted by the method described by Bligh and Dyer [3] and stored at −20°C. Lipids (1 mg) were suspended in 0.5 ml 50 mM potassium phosphate buffer (pH 7.2) containing 2% sodium cholate, 1% sodium deoxycholate (lipid/detergent mixture), H protein (50—100 μg) and sucrose (final 0.25 M). Then the mixture was dialysed overnight against 50 mM potassium phosphate buffer (pH 8.3 for high pH vesicles, pH 7.2 for neutral pH ones, pH 6.5 for low pH ones). The dialysate (vesicles) was centrifuged at 100 000 × *g* for 30 min and the precipitate was suspended in 0.3 M sucrose/15 mM $MgCl_2$.

Transport energized by Na^+ gradient. The vesicles (neutral pH vesicles) were suspended in 0.45 ml a solution comprising (final concentrations) 50 mM potassium-phosphate buffer (pH 7.2)/15 mM $MgCl_2$/0.25 M sucrose/25 μM of L-[U-^{14}C]glutamate. After incubation for 2 min at 27°C, the reaction was started by the addition of 0.05 ml 0.5 M NaCl solution.

Transport energized by pH gradient. The vesicles (high pH vesicles) were suspended in 0.5 ml of solution comprising (final concentrations) 50 mM potassium phosphate buffer (pH 7.2)/15 mM $MgCl_2$/0.25 M sucrose/50 μM of L-[U-^{14}C]glutamate. After incubation for 2 min at 27°C, the reaction was started by the addition of 10 μl 1 M HCl.

Transport energized by H^+-diffusion potential. The vesicles formed at pH 6.5 (low pH vesicles) were suspended in 0.5 ml of a solution comprising 50 mM potassium phosphate buffer (pH 8.3)/15 mM $MgCl_2$/0.25 M sucrose/50 μM L-[U-^{14}C]glutamate. After incubation for 30 s at 27°C, the reaction was started by the addition of 2 μl mM CCCP (carbonylcyamide *m*-chlorophenylhydrozone). Transport driven by membrane potential via K^+ diffusion was studied as described previously [2].

In all assays, a 50-μl portion was filtered through membrane filters (Sartorius, 0.45 μm pore size) and washed with 0.3 M sucrose/15 mM $MgCl_2$. The filters were dried and their radioactivities were counted in a gas-flow counter. The activity was expressed as initial rates of transport which were determined from the initial linear portion of the curves.

Proteins were determined as described by Lowry et al. [4].

Monensin was a generous gift of Dr. N. Ohtake of this Institute.

Results

The vesicles formed at pH 7.2 were suspended in varying concentrations of NaCl, and glutamate transport activities were assayed. As shown in Fig. 1, a chemicals gradient of Na^+ could drive glutamate transport and the transport continued for 5 min at quite constant rates.

The effect of Na^+ on the transport was studied on three kinds of vesicle: the

vesicles were formed by dialysis in the solution of (1) 100 mM sucrose ('sucrose-loaded vesicles'); (2) 50 mM potassium phosphate buffer, pH 7.2 ('potassium phosphate-loaded'); (3) 50 mM sodium phosphate buffer, pH 7.2 ('sodium phosphate-loaded'). These vesicles were suspended in the presence or absence of Na^+ (50 mM sodium phosphate buffer, pH 7.2) and the glutamate transport activities were estimated. As shown in Fig. 2, in the presence of Na^+, transport activity was found in sucrose- and potassium phosphate-loaded vesicles but not in the sodium phosphate-loaded. These results indicate that glutamate could accumulate when a Na^+ gradient across the membrane was imposed.

Na^+-dependent transport of glutamate was inhibited almost completely by 1 μg of monensin and was not influenced by $2 \cdot 10^{-5}$ M CCCP.

Effect of Na^+ concentrations on the rates of glutamate transport was examined. The transport was energized again by Na^+ gradient and the rates were determined by varying concentrations of NaCl added in the reaction mixture. As shown in Fig. 3, the maximum effect of Na^+ on the transport is achieved at a concentration of about 40 mM, while the apparent K_m for Na^+ is approximately 18 mM (Fig. 3, insert).

Kahane et al. [5] have shown that increase in Na^+ concentration decreased the apparent K_m for glutamate in the Na^+-dependent glutamate transport in *E. coli*. Therefore, we have also examined the effect of Na^+ on our glutamate transport system. The kinetics of the effect of Na^+ on glutamate transport was studied in an experiment in which glutamate concentration was varied in the presence of 50 and 4 mM NaCl. A Lineweaver-Burk plot of the data (Fig. 4)

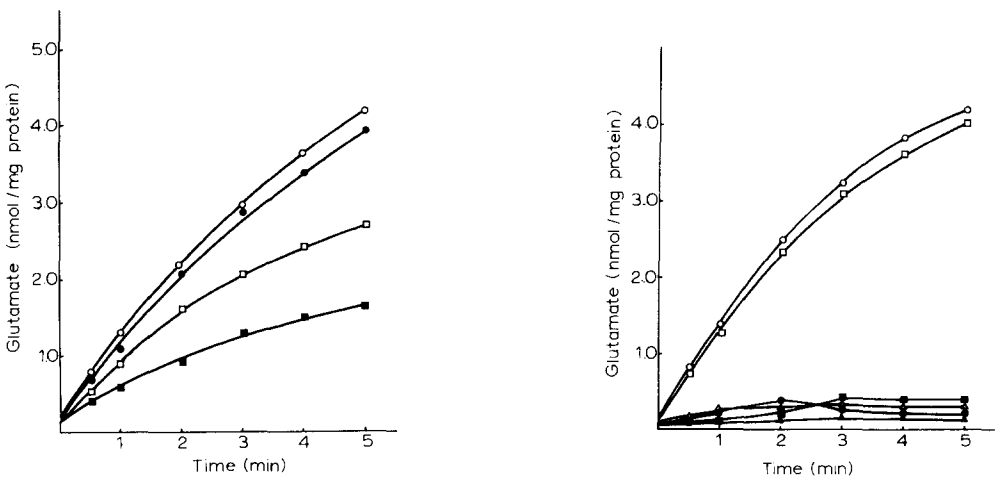

Fig. 1. Sodium dependency of glutamate transport in the vesicles formed by H protein. Na^+ gradient was produced by the addition of NaCl to the reaction mixture. Membrane vesicles formed by dialysis in 50 mM potassium-phosphate buffer (pH 7.2) were suspended in 50 mM potassium-phosphate buffer (pH 7.2) containing 50 mM (○) 25 mM (●) 10 mM (□) and 5 mM (■) NaCl, respectively.

Fig. 2. Effect of Na^+ gradient on the glutamate transport of the vesicles formed by H protein. 100 mM sucrose-loaded (circles), 50 mM potassium-phosphate-loaded (squares). 50 mM sodium phosphate-loaded (triangles) were suspended in the presence of 50 ml NaCl (open symbols) or in its absence (closed symbols).

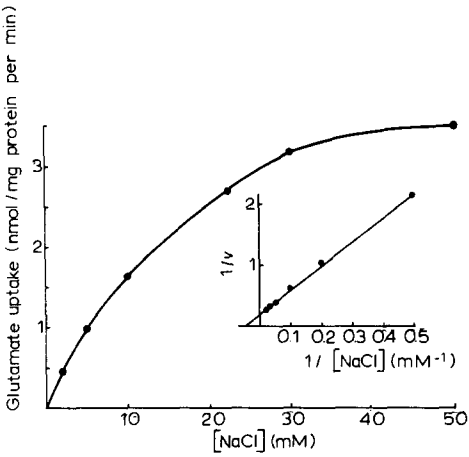

Fig. 3. Effect of NaCl concentrations on glutamate transport in the vesicles formed by H protein. Vesicles formed in 50 mM potassium-phosphate buffer (pH 7.2) were suspended in 50 mM potassium-phosphate buffer (pH 7.2) containing varying concentrations of NaCl.

shows that Na^+ increases the affinity for glutamate, but does not affect the transport activity (K_m for glutamate in the presence of 50 mM NaCl was 8 μM; on the other hand, K_m in the presence of 4 mM NaCl was about 50 μM). These results indicate that H protein contains carrier which mediates Na^+/glutamate symport.

We have studied next the glutamate transport driven by pH gradient. The vesicles formed in pH 8.3 (high pH vesicles) were suspended in 50 mM potassium phosphate buffer (pH 7.2)/15 mM $MgCl_2$/0.25 M sucrose/50 μM [^{14}C]-glutamate. After incubation for 2 min at 27°C, 15 μl of 1 M HCl was added and the accumulation of glutamate in the vesicles was assayed. As shown in Fig. 5,

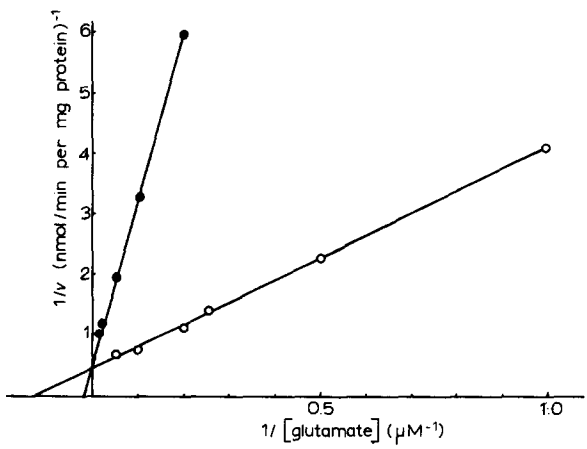

Fig. 4. Effect of NaCl concentrations on the kinetics of glutamate transport. Vesicles formed in 50 mM potassium phosphate buffer (pH 7.2) were suspended in 50 mM potassium phosphate buffer (pH 7.2) containing 4 mM (●) or 50 mM (○) NaCl and varying concentrations of glutamate.

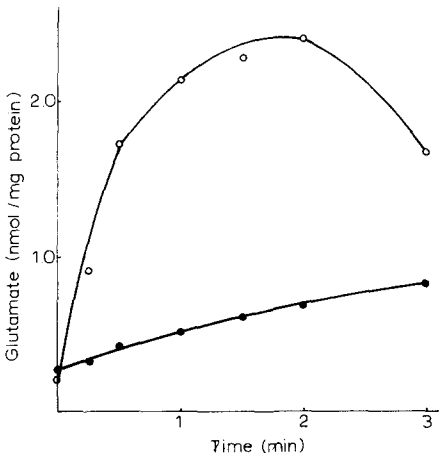

Fig. 5. Glutamate transport driven by pH gradient and effect of CCCP on the transport. Vesicles formed in 50 mM potassium phosphate buffer (pH 8.3) were suspended in 50 mM potassium phosphate buffer (pH 7.2). The reaction was started by the addition of HCL in the presence (●) of 20 μM CCCP or in its absence (○).

glutamate was accumulated in the vesicles by the addition of HCl in the external medium. The accumulation was blocked by 10 μM CCCP as shown in the Fig. 5. These results indicate that glutamate is transported also by pH gradient, and carrier which mediates H^+/glutamate symport is present in H proteins.

An apparent K_m value for glutamate in pH gradient-driven glutamate transport was determined. A Lineweaver-Burk plot of the data (Fig. 6) shows that

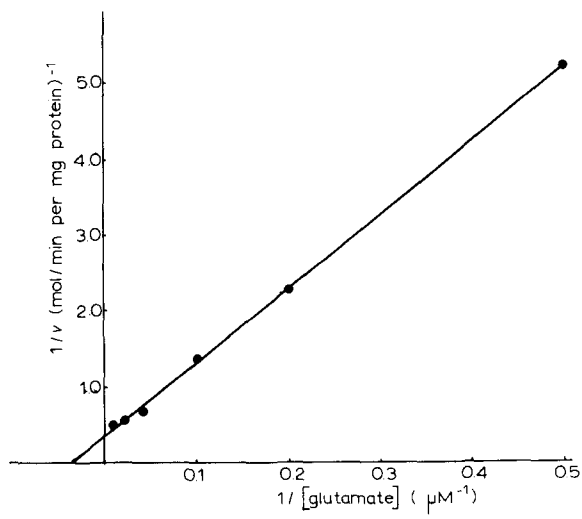

Fig. 6. Lineweaver-Burk plot of glutamate transport driven by pH gradient. Experimental conditions were the same as those described in Fig. 5.

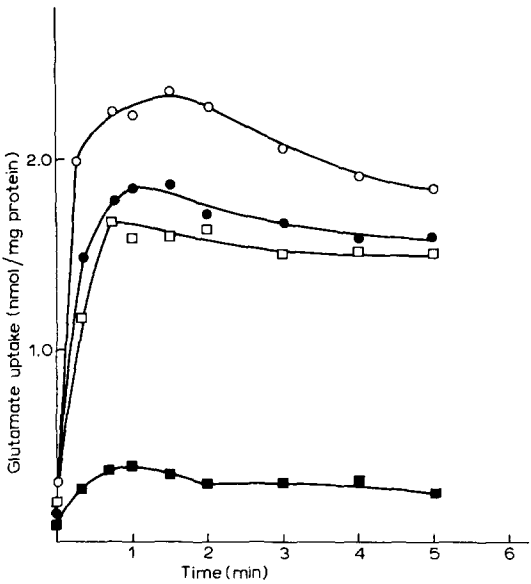

Fig. 7. Glutamate transport driven by K^+-diffussion membrane potential. K^+-loaded vesicles (0.5 M potassium phosphate buffer) were suspended in Tris-HCl buffer (pH 7.2) and the reaction was started by the addition of 1 μg of valinomycin. Transport activities were observed in 10 μM (squares) and 50 μM (circles) glutamate in the absence (closed symbols) and presence (open symbols) of 100 mM NaCl.

K_m for glutamate was about 25 μM, which is larger than that of Na^+ dependent glutamate transport system.

Glutamate transport was also observed on the vesicles energized by membrane potential created by K^+- or H^+-diffusion.

An example of the transport driven by K^+-diffusion potential is shown in Fig. 7. In the figure, we show the transport activities in glutamate concentrations of 10 and 50 μM. In the absence of Na^+, 10 μM of glutamate was not sufficient to drive transport carrier and the activity was found only if 50 μM of glutamate were present. This is because of the high K_m value for glutamate of the H^+-dependent transport system. On the other hand, in the presence of Na^+, transport can be observed at both (10 and 50 μM) glutamate concentrations.

Different pH-dependencies between these two transport systems were also observed. Acitivyt of Na^+-dependent transport activity had a rather sharp pH optimum at around pH 7.2. On the other hand, the H^+-dependent transport was active rather in acidic pH, and significant activities were found even at pH 4.5. The optimum pH was around 6.3.

Discussion

H protein is membrane protein which is not integrated into membrane and is accumulated in membrane fraction. Therefore, H protein can easily be isolated from the membrane fractions [2]. Isolated H proteins can be constituted into membrane vesicles binding with lipid and the constituted mem-

branes show activities of NADH, succinate oxidases and active transport of alanine and proline.

In the present paper, we have shown that H protein contained two kinds of glutamate transport system: the one is Na^+-dependent and the other is H^+-dependent. Apparent K_m for glutamate of these two systems were different. Different pH dependencies between two systems were also observed.

Calculations show that the intravesicular concentrations of glutamate of H^+ dependent transport was about 1.2 mM, therefore, glutamate was concentrated about 24-fold inside the vesicles. In the case of Na^+ dependent ones, glutamate was concentrated about 75-fold inside the vesicles.

Recently, Na^+/H^+ antiport was demonstrated in the membranes of *Escherichia coli* [6,7] and *Halobacterium halobium* [8]. We have also tried to demonstrate the antiport in H proteins. Na^+-loaded vesicles was suspended in Na^+-free medium and determined the ΔpH produced according to the method described by Schuldiner et al. [7]. Protoplast membrane of *B. substilis* could produce a change in pH but artificial membrane vesicles formed by H protein could not produce any detectable pH difference across the membranes. If H protein contains Na^+/H^+ antiport, the antiport may be unstable and the activity may be lost during isolation and constitution.

Glutamate transport system has been studied in *E. coli* [5,9,10], *H. halobium* [8] and *Staphylococcus aureus* [11]. The systems found in *E. coli* and *H. halobium* are Na^+ dependent but the system in *S. aureus* is H^+ dependent. H protein of *B. subtilis* contains these two kinds of glutamate transport system.

Glutamate transport carriers in H protein can easily be solubilized by using area as that of alanine which is already purified and characterized [12]. Glutamate transport carriers may be purified in the near future from H protein.

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