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Mechanism of Glutamate Transport in *Escherichia coli* B. 1. Proton-Dependent and Sodium Ion Dependent Binding of Glutamate to a Glutamate Carrier in the Cytoplasmic Membrane†

Tsutomu Fujimura, Ichiro Yamato, and Yasuhiro Anraku*

ABSTRACT: Specific binding of glutamate to its carrier was investigated by using cytoplasmic membrane vesicles prepared from *Escherichia coli* B. The binding activity was specifically affected by the Na^+ and H^+ concentrations of the medium. Cytoplasmic membrane vesicles from the mutant strain 36-39 that is defective in the Na^+-dependent glutamate transport system showed no binding of glutamate. Addition of the protonophore uncoupler 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile or carbonyl cyanide *m*-chlorophenylhydrazone, or the ionophore monensin or nigericin, did not inhibit the binding, indicating that the binding reaction is not energy dependent. The parameters of binding were determined in reaction media with various combinations of H^+ and Na^+ concentrations. The maximum number of binding sites was constant and determined to be 70 pmol/mg of membrane protein, irrespective of the concentrations of H^+ and Na^+ in the medium. The apparent dissociation constant, however, was greatly affected by changes in the concentrations of both H^+ and Na^+, in such a way that it was expressed by a linear combination of the reciprocals of the H^+ and Na^+ concentrations. The characteristics of binding can be explained best by supposing that glutamate can bind only to a H^+/Na^+/carrier complex that is formed by random binding of H^+ and Na^+ to the unloaded carrier. The physiological role of this elementary binding reaction and of this quaternary complex as an active intermediate in the process of glutamate transport is discussed.

Studies on the energetics of active solute transport in bacteria have progressed extensively since proposition of the chemiosmotic hypothesis (Mitchell, 1966, 1967; Rosen & Kashket, 1978). Carrier proteins are recognized to function mostly as secondary chemiosmotic pumps that utilize an electrochemical gradient of proton or sodium ion as a driving force. However, the molecular mechanism of active transport catalyzed by syn-coupled and anti-coupled reactions remains unsolved (Mitchell, 1967; Rosen & Kashket, 1978).

Frank & Hopkins (1969) demonstrated that glutamate transport in *Escherichia coli* B and its derivatives is stimulated by Na^+ and that this stimulation is due to an increase in the affinity for substrate. Glutamate transport activity was retained on membrane vesicles, and a periplasmic binding protein was not intimately involved in the transport process (Minor & Frank, 1974). Later, two groups of investigators proposed the mechanism of a Na^+/glutamate symport (Hasan & Tsuchiya, 1977; Tsuchiya et al., 1977; MacDonald et al., 1977), based on the observations that a chemical gradient of Na^+ imposed directly on intact cells and membrane vesicles caused the accumulation of glutamate.

Our current interest in active transport has been focused specifically on the initial step of the H^+/substrate symport reaction. Thus, we have investigated the specific binding of proline to its carrier in membrane vesicles of *Escherichia coli* and the effects of H^+ and/or Na^+ on the binding (Amanuma et al., 1977; Anraku, 1982; Motojima et al., 1979; T. Mogi and Y. Anraku, unpublished results). The results of these studies and of the present work indicated that co-ions (H^+ and Na^+) initially bind to a carrier that catalyzes a syn-coupled reaction and that the binary or ternary complex of the carrier with co-ions then binds substrate to form an intermediate for translocation across the cytoplasmic membrane.

In this paper, we demonstrate the specific stimulatory effect of H^+, in addition to Na^+, on glutamate binding and propose a model of glutamate binding to the carrier. In the following paper (Fujimura et al., 1983), we propose a model of glutamate transport based on the binding model and describe the mechanism of syn-coupled transport of $Na^+/H^+/Glu^-$ via the glutamate carrier.

Experimental Procedures

Bacterial Strains. Escherichia coli B (wild strain) and its derivative, strain 36-39, were obtained from T. Tsuchiya (Okayama University, Okayama). Strain 36-39 is resistant to methyl DL-α-glutamate and shows low activity of Na^+-independent glutamate uptake (Tsuchiya et al., 1977). These strains were grown in Na^+-free medium B7 (Frank & Hop-

† From the Department of Biology, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan. *Received September 29, 1982.* This research was supported in part by a Grant-in-Aid for a Special Research Project from the Ministry of Education, Science, and Culture of Japan and a grant from the Toray Science Foundation of Japan.

0006-2960/83/0422-1954$01.50/0

kins, 1969) supplemented with 0.4% glycerol and 20 mM NH_4Cl.

Preparation of Cytoplasmic Membrane Vesicles. Cytoplasmic membrane vesicles were prepared by the method of Yamato et al. (1975) except that potassium ethylenediaminetetraacetic acid (K^+-EDTA)[1] was used in place of Na^+-EDTA. Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Binding Assay. A centrifugation method (Kennedy et al., 1974) was employed to measure the binding of glutamate to cytoplasmic membrane vesicles. The standard assay medium (1 mL) contained 50 mM Mes–KOH (pH 6.1), 100 mM NaCl, cytoplasmic membrane vesicles (1.3–2.0 mg of protein), and [^{14}C]glutamic acid (0.5 μM) in a centrifuge tube. Unless otherwise noted, the cytoplasmic membrane vesicles used were from strain B. The control contained 1 mM unlabeled glutamic acid in addition to the constituents of the standard assay medium. Other additions were as indicated in the text.

The binding reaction was started by adding [^{14}C]glutamic acid to assay medium in which cytoplasmic membrane vesicles had been incubated for 10 min at 25 °C. Under standard conditions, the amounts of glutamate bound were determined to be 27.6, 31.2, 31.2, and 29.1 pmol/mg of membrane protein after incubation for 3, 10, 20, and 40 min, respectively, at 25 °C, indicating that the binding reached equilibrium within 10 min. Thus, after incubation for 20 min, the vesicles were precipitated by centrifugation at 200000*g* for 20 min at 15 °C.

Under standard conditions, the amount of glutamate taken into the vesicles by the process of facilitated diffusion, if any, was negligibly small since it could be maximally 1.1 pmol/mg of membrane protein when the internal water space of the membrane vesicles was assumed to be 2.2 μL/mg of membrane protein (Fujimura et al., 1983): This value was only one-thirtieth of the observed amount of glutamate bound to cytoplasmic membrane vesicles.

For estimation of the free substrate concentration, the radioactivity of an aliquot of the supernatant (100 μL) was counted. The rest of the supernatant was carefully removed, and the residual solution in the tube was wiped off. Then, the precipitate was dissolved in 1 mL of 5% Triton X-100, and its radioactivity was counted after mixing it with 6 mL of a Triton X-100–toluene counting solution (Patterson & Greene, 1965). From the difference in the radioactivities of the precipitates in the sample and control tubes, the amount of glutamate specifically bound to the saturable binding sites of carrier in the membranes was calculated.

Analysis of Radioactive Material(s) Bound to Cytoplasmic Membrane Vesicles. Radioactive material(s) bound to the vesicles was (were) identified as glutamate by thin-layer chromatography. After the reaction in the standard assay medium, the vesicles were precipitated by centrifugation, and the supernatant was carefully removed. The precipitate was suspended in 50 μL of deionized water, and 5 μL of trichloroacetic acid (50% w/v) and 10 μL of unlabeled glutamic acid (1.4 mM) were added. The mixture was heated in a boiling water bath for 5 min and then cooled. Then 10 μL of the supernatant (8×10^3 cpm) was spotted on a silica gel plate (silica gel 60, Merck) and developed with 80:20:20 (v/v) 1-butanol:acetic acid:water. A width of 1 cm of the silica was then scraped off, and its radioactivity was determined in a liquid scintillation counter.

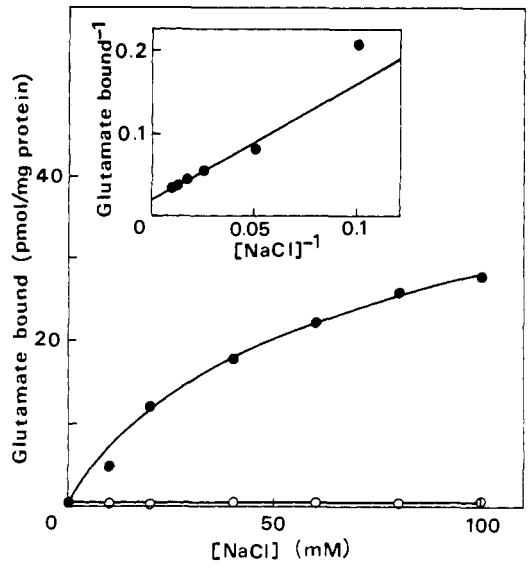

FIGURE 1: Effect of Na^+ concentration on glutamate binding. Glutamate binding was measured under standard assay conditions. The cytoplasmic membrane vesicles used were prepared from *E. coli* B (●) or from strain 36-39 (○). A double-reciprocal plot of the data on strain B vesicles is shown in the inset.

Chemicals. Valinomycin and Mes were purchased from Sigma Chemical Co. L-[U-^{14}C]Glutamic acid (285 mCi/mmol) was from the Radiochemical Centre, Amersham. SF6847 was kindly supplied by Dr. Y. Nishizawa, Sumitomo Chemicals, Osaka, and nigericin by Dr. H. Maruyama, Roche Institute of Japan, Kanagawa. Monensin was provided by Lilly Laboratories, Indianapolis, IN. Other chemicals were standard commercial products of analytical grade.

Results

Na^+-Dependent Binding of Glutamate. Cells of *E. coli* B possess an Na^+-dependent glutamate transport system (Frank & Hopkins, 1969). Cytoplasmic membrane vesicles prepared from wild-type strain B showed Na^+-dependent glutamate binding as seen in Figure 1. Cytoplasmic membrane vesicles prepared from mutant strain 36–39, which has no Na^+-dependent glutamate transport system, showed no binding activity. A double-reciprocal plot of the amount of glutamate bound vs. the Na^+ concentration in the medium was linear as indicated in the inset of Figure 1, suggesting that a single species of glutamate carrier is responsible for this binding and requires 1 mol of Na^+ to bind 1 mol of glutamate. The glutamate bound to the membrane vesicles was examined by thin-layer chromatography as described under Experimental Procedures. More than 90% of the radioactivity bound to the membranes was recovered as glutamic acid, indicating that the glutamate bound to the membrane vesicles did not undergo metabolic alteration.

The binding activity was not inhibited at all by the addition of the uncoupler SF6847 (0.5 μM) or CCCP (10 μM), or the ionophore monensin (5 μM) or nigericin (0.5 μM) that catalyzes an electrically neutral exchange of Na^+ or K^+ with a proton. Simultaneous addition of SF6847 (0.5 μM) and monensin (5 μM) did not inhibit the activity either. These results indicate that the binding to the cytoplasmic membranes was not due to energy-dependent uptake of glutamate into the vesicles.

The effects of monovalent cations on glutamate binding were examined. Only Na^+ was effective whereas K^+ and Rb^+ were

[1] Abbreviations: SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; K_d, dissociation constant of binding; $[BS]_{max}$, maximum number of binding sites; C_t, total amount of glutamate carrier.

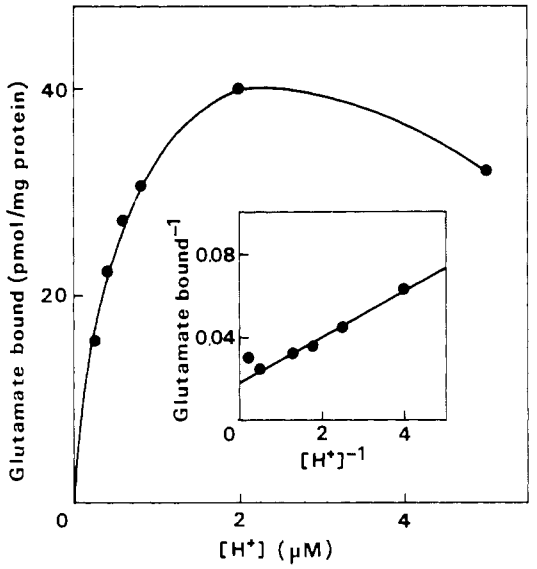

FIGURE 2: Effect of H^+ concentration on glutamate binding. Glutamate binding was measured as described under Experimental Procedures. The assay mixture contained 50 mM Mes–KOH (pH 5.3–6.6), 60 mM NaCl, 1 μM [^{14}C]glutamic acid, and cytoplasmic membrane vesicles (1.9 mg of protein) prepared from *E. coli* B. A double-reciprocal plot of the data is shown in the inset.

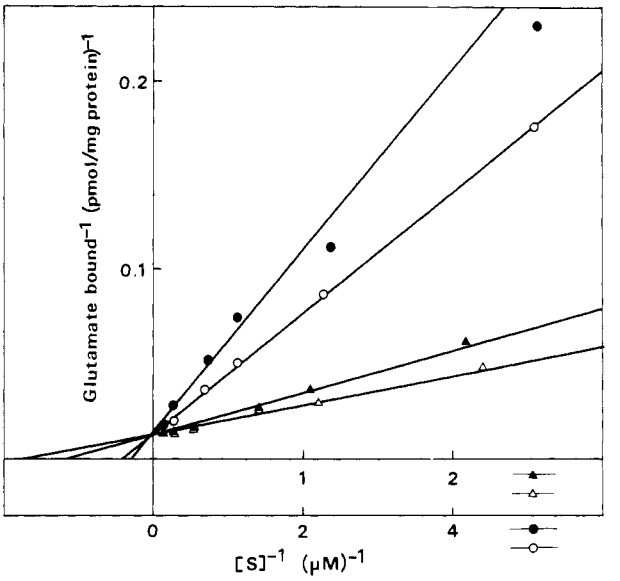

FIGURE 3: Double-reciprocal plots of glutamate binding at pH 6.4. The assay mixture contained 50 mM Mes–KOH (pH 6.4), [^{14}C]-glutamic acid (0.2–20 μM), cytoplasmic membrane vesicles (1.9 mg of protein), and a mixture of NaCl and KCl (final concentration of Cl^- 100 mM). The concentrations of Na^+ used were 15 (●), 25 (○), 40 (▲), and 100 mM (△).

not. Lithium ion, which has been reported to replace Na^+ as substrate in transport by the Na^+/H^+ antiport system (Schuldiner & Fishkes, 1978; Beck & Rosen, 1979) and in thiomethyl galactoside cotransport by the melibiose transport system (Lopilato et al., 1978) in *E. coli*, was ineffective. We have also found that Li^+ alone does not stimulate glutamate transport in *E. coli* B, in agreement with the observation of Hasan & Tsuchiya (1977). All the results indicate that the glutamate carrier in nonenergized membranes can bind glutamate specifically in the presence of sodium ion.

H^+ Dependence of Glutamate Binding. We further found that this Na^+-dependent glutamate binding is affected by the pH of the assay medium. Figure 2 shows the H^+ dependence of glutamate binding in the presence of 60 mM NaCl. Similar H^+ dependence of binding was observed in the presence of NaCl from 15 to 100 mM. The decrease of the binding activity at 5 μM H^+ (pH 5.3) was probably due to a nonspecific effect of H^+ on the carrier, because aggregation of membranes was observed at pH 5.3 or less. A double-reciprocal plot of the amount of glutamate bound vs. the H^+ concentration in the medium gave a straight line (Figure 2, inset), which suggests that the glutamate carrier requires 1 mol of H^+ to bind 1 mol of glutamate.

Effects of Na^+ and H^+ on the Parameters of Binding. The effects of Na^+ and H^+ on glutamate binding to carriers were further investigated by determining the binding parameters of the maximum number of binding sites ($[BS]_{max}$) and the apparent dissociation constant (K_d). The parameters were obtained from double-reciprocal plots of glutamate binding in media with various concentrations of Na^+ at a constant ionic strength. Figure 3 shows results of a reaction carried out at pH 6.4. The double-reciprocal plot gave a straight line, irrespective of the concentration of NaCl. Similar experiments were carried out in media of pH 5.3, 5.7, 6.1, and 6.6, and the K_d values obtained were plotted as shown in Figure 4. We found that the $[BS]_{max}$ value was constant within experimental error, irrespective of the Na^+ and H^+ concentrations, and it was determined to be about 70 pmol/mg of membrane protein.

K_d values, however, decreased greatly with an increase in the Na^+ concentration (Figure 3). More specifically, we found

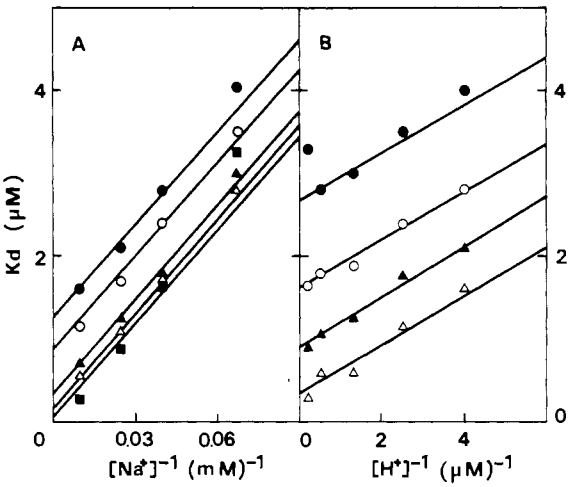

FIGURE 4: Effects of Na^+ and H^+ concentrations on the apparent dissociation constant (K_d) of glutamate binding. The K_d values were obtained graphically from Figure 3 and other similar experiments but with different concentrations of H^+ and Na^+. (A) The concentration of Na^+ was changed at constant pH values of 6.6 (●), 6.4 (○), 6.1 (▲), 5.7 (△), and 5.3 (■). (B) The pH was changed at constant concentrations of NaCl of 15 (●), 25 (○), 40 (▲), and 100 mM (△).

that the K_d values changed as a linear function of the reciprocal of the Na^+ concentration. The slope of the straight line was constant irrespective of the pH of the reaction medium, and the value of $K_{d\,[Na^+]\to\infty}$ decreased with an increase of the H^+ concentration (Figure 4A). Similarly, replots of K_d values against the reciprocals of H^+ concentrations fell on a straight line. Here again, the slope was constant, and $K_{d\,[H^+]\to\infty}$ values decreased with increasing Na^+ concentration (Figure 4B). The $K_{d\,[Na^+]\to\infty}$ and $K_{d\,[H^+]\to\infty}$ values were obtained graphically from panels A and B, respectively, of Figure 4 and then replotted against the reciprocals of H^+ and Na^+ concentrations, respectively (Figure 5). In both cases, the lines appeared to cross close to the origin. These results indicate that the K_d value is affected equally and solely by the concentrations of H^+ and Na^+. In other words, the glutamate carrier absolutely requires both Na^+ and H^+ for substrate binding, and its ap-

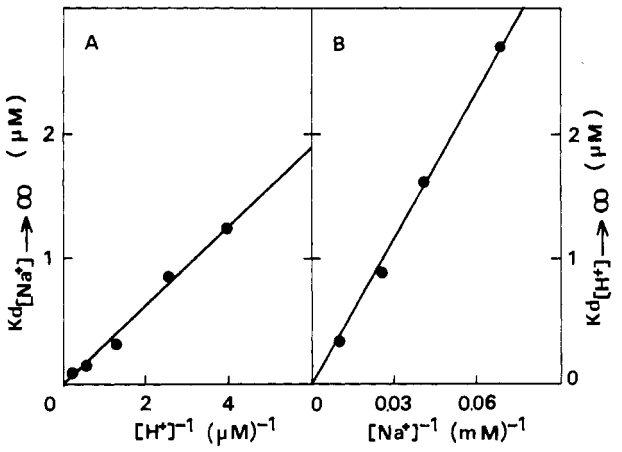

FIGURE 5: Secondary plots of the dissociation constant. (A) K_d values at infinite concentrations of Na^+ in medium of the pH indicated were obtained graphically from Figure 4A and then plotted against the reciprocals of the H^+ concentrations. (B) K_d values at infinite concentrations of H^+ in the medium with the NaCl concentration indicated were obtained from Figure 4B and plotted as indicated.

parent dissociation constant in a specified condition is given by a linear combination of the reciprocals of the Na^+ and H^+ concentrations.

From the slopes of the lines in Figure 5A,B, the dissociation constant is given empirically by

$$K_d = \frac{0.32}{[H^+]} + \frac{40}{[Na^+]} \quad (1)$$

where the dimensions of molarity for K_d, $[H^+]$, and $[Na^+]$ are micromolar, micromolar, and millimolar, respectively.

Discussion

We found that the glutamate carrier specifically requires both Na^+ and H^+ for binding of glutamate. Li^+ did not replace Na^+ for this reaction, and the cation specificity was the same as that of glutamate transport in intact cells or cytoplasmic membrane vesicles driven by artificially imposed chemiosmotic energy (Fujimura et al., 1983). Furthermore, the Na^+- and H^+-dependent binding (Figures 1 and 2) was shown to be due to a single species of glutamate carrier that catalyzes Na^+- and H^+-dependent glutamate transport in intact cells (unpublished results) and in vesicles (Fujimura et al., 1983).

Stoichiometric binding analysis (Figures 3–5) indicated clearly that (1) the $[BS]_{max}$ value is constant irrespective of the Na^+ and H^+ concentrations of the medium and that (2) the K_d values are affected greatly by the concentrations of both Na^+ and H^+ in the medium and can be expressed empirically by a function of a linear combination of the reciprocals of the Na^+ and H^+ concentrations as in eq 1, where the two constants given were obtained graphically from Figure 5.

To explain all these results and the mechanism of elementary reactions reflecting the Na^+ and H^+ dependence of glutamate binding to the carrier, we propose the model shown in Figure 6, where the following assumptions are adopted: (1) The glutamate carrier requires 1 mol of Na^+ and 1 mol of H^+ to bind 1 mol of glutamate. (2) Binding of Na^+ and H^+ to an unloaded carrier is random, but only the ternary complex carrier/Na^+/H^+ (CNa^+H^+) is active for glutamate binding. Then, the amount of glutamate bound to the carrier ([BS]) given by these assumptions is

$$[BS] = [CH^+Na^+S^-] = \frac{C_t[S^-]}{K_d^{true} + [S^-]} \quad (2)$$

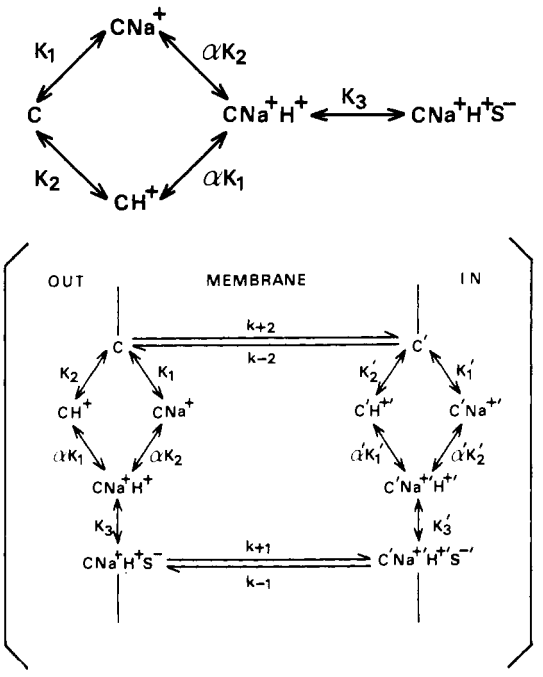

FIGURE 6: Model of glutamte binding. C and S^- represent the glutamate carrier and glutamate, respectively. K_1, K_2, and K_3 are the dissociation constants of the elementary reactions indicated in the figure. The CNa^+H^+ complex can be formed in two dependent ways: $C \leftrightarrow CNa^+ \leftrightarrow CNa^+H_+$ and $C \leftrightarrow CH^+ \leftrightarrow CNa^+H^+$. Therefore, $K_1(\alpha K_2) = K_2(\alpha K_1)$, so α becomes a single value. Inset: This model postulates that binding of glutamate to the carrier, as shown in Figure 6, occurs symmetrically at the external and internal surfaces of the membrane. K_1, K_2, and K_3 and K_1', K_2', and K_3' are the dissociation constants at the external and internal surfaces of the membrane, respectively. k_{+1}, k_{-1} and k_{+2}, k_{-2} are the respective permeability coefficients in the forward and reverse directions of the $CNa^+H^+S^-$ complex and of the unloaded carrier, respectively. K_a, an equilibrium constant for the interconversion of the internal and external pools of the unloaded carrier, is defined as k_{+2}/k_{-2}.

where C_t is the total amount of the carrier and $[S^-]$ the concentration of free substrate. K_d^{true} is defined as

$$K_d^{true} = \frac{\alpha K_1K_2K_3}{[H^+][Na^+]} + \frac{\alpha K_2K_3}{[H^+]} + \frac{\alpha K_1K_3}{[Na^+]} + K_3 \quad (3)$$

To explain our experimental observations within the framework of our model, we have to adopt two further assumptions: (3) The concentration of the unloaded carrier in the cytoplasmic membrane is negligibly low, and its affinities for Na^+ and H^+ are very high. (4) The dissociation constant K_3 of the ternary complex CNa^+H^+ for glutamate binding should be small and must be smaller than 10^{-7} M. In other words, K_3, as defined by eq 3, is graphically represented as the intercepts of the y axes in panels A and B of Figure 5, both of which are close to zero. This observation can be explained by assuming that K_3 is much smaller than 10^{-7} M.

Then, eq 2 approximates to

$$[BS] = \frac{C_t[S^-]}{\alpha K_2K_3/[H^+] + \alpha K_1K_3/[Na^+] + [S^-]} \quad (4)$$

Hence, the apparent dissociation constant, K_d, for glutamate is represented by a linear combination of the reciprocals of Na^+ and H^+ concentrations in the medium as in eq 5.

$$K_d = \frac{\alpha K_2K_3}{[H^3]} + \frac{\alpha K_1K_3}{[Na^+]} \quad (5)$$

Equation 5 is identically coordinated to eq 1, which was ob-

tained experimentally. Moreover, eq 4 shows that the maximum number of binding sites is equal to C_t and independent of the concentrations of both Na^+ and H^+.

We also examined other models such as the sequential order binding of H^+ or S^- to the unloaded carrier ($C \leftrightarrow CH^+ \leftrightarrow CH^+Na^+ \leftrightarrow CH^+Na^+S^-$; $C \leftrightarrow CH^+ \leftrightarrow CH^+S^- \leftrightarrow CH^+S^-Na^+$; $C \leftrightarrow CS^- \leftrightarrow CS^-H^+ \leftrightarrow CS^-H^+Na^+$) and models such as the sequential order binding of Na^+ or S^- to the unloaded carrier similar to those shown above. We found that all these models did not explain the H^+- and Na^+-dependent changes of the apparent dissociation constant given in eq 1. For example, in the model

$$C \overset{K_4}{\leftrightarrow} CH^+ \overset{K_5}{\leftrightarrow} CH^+Na^+ \overset{K_6}{\leftrightarrow} CH^+Na^+S^-$$

where K_4, K_5, and K_6 are the dissociation constants of the reactions indicated, K_d^{true} is expressed as $K_d^{true} = (K_4/[H^+] + 1)K_5K_6/[Na^+] + K_6$.

Glutamate predominantly exists in a negatively charged form in medium of pH 4–9. Therefore, a model in which undissociated glutamic acid or sodium glutamate is the actual substrate for binding is unlikely and was excluded. In the model

$$C \overset{K_7}{\leftrightarrow} CNa^+ \qquad S^- \overset{K_8}{\leftrightarrow} S^-H^+$$
$$CNa^+ + S^-H^+ \overset{K_9}{\leftrightarrow} CNa^+S^-H^+$$

where K_7, K_8, and K_9 are the dissociation constants of the reactions indicated, K_d^{true} can be expressed as $K_d^{true} = (K_7/[Na^+] + 1)K_8K_9/[H^+]$.

The topology of the carrier in the membrane is indicated in the inset of Figure 6. Under the standard assay conditions employed in this study, the internal fluid of the vesicles was equilibrated with the external medium. Therefore, glutamate could gain access to the Na^+- and H^+-loaded carrier from the internal space of the vesicles.[2] If it is assumed that the carrier protein is symmetrically located in the membrane, the quaternary complex $CNa^+H^+S^-$ will be formed at both the internal and external surfaces (Figure 6, inset). The amount of glutamate bound to the carrier ([BS]) can be calculated by using eq 6

$$[BS] = C_t[S^-]/\left\{\frac{1}{\beta}\left[\frac{1+K_a}{[H^+][Na^+]} + \frac{1}{[H^+]}\left(\frac{1}{K_1}+\frac{K_a}{K_1'}\right) + \frac{1}{[Na^+]}\left(\frac{1}{K_2}+\frac{K_a}{K_2'}\right) + \left(\frac{1}{\alpha K_1K_2} + \frac{K_a}{\alpha' K_1'K_2'}\right)\right] + [S^-]\right\} \quad (6)$$

where

$$\beta = \frac{1}{\alpha K_1K_2K_3} + \frac{K_a}{\alpha' K_1'K_2'K_3'}$$

and K_1, K_2, K_3 and K_1', K_2', K_3' are the dissociation constants at the external and internal surfaces, respectively. For the definition of K_a, see the legend to Figure 6.

[2] Seventy percent of the cytoplasmic membrane vesicles are right side out, and the rest are inside out or unsealed (Yamato et al., 1978). Under the standard experimental conditions used, the amount of glutamate bound to the carrier was not affected by the proportions of membrane vesicles with different topologies.

If assumptions similar to those used for approximating eq 2 to eq 4 are made (that the concentration of the unloaded carrier is low and that the dissociation constants K_3 and K_3' are negligibly small), eq 6 can be simplified to eq 7.

$$[BS] = \frac{C_t[S^-]}{\frac{1}{\beta}\left[\frac{1}{[H^+]}\left(\frac{1}{K_1}+\frac{K_a}{K_1'}\right) + \frac{1}{[Na^+]}\left(\frac{1}{K_2}+\frac{K_a}{K_2'}\right)\right] + [S^-]} \quad (7)$$

Equation 7 exhibits exactly the same dependence on Na^+ and H^+ in the binding reaction as eq 1 and 4. Moreover, if the dissociation constant K_1 is equal to K_1' and K_2 is equal to K_2', eq 7 can be simplified to eq 4.

Recently, Wright et al. (1981) reported that the binding of β-galactoside and the proton to *E. coli* lactose carrier occurred independently in a random order. Their proposal stemmed from the following observations: (1) The K_d value for lactose or *p*-nitrophenyl α-galactoside of the lactose carrier became nearly constant between pH 5.5 and 7.6. (2) A saturable amount of β-D-galactosyl-1-thio-β-D-galactoside did not induce proton binding to the carrier between pH 6.11 and 6.52. However, if the K_d for the proton of the unloaded carrier were 10^{-8} M or less, their results would not necessarily rule out other reaction models, such as an ordered binding mechanism where the proton binds first and leaves last (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979) or a random-order mechanism where β-galactoside and the proton bind independently of one another (Page & West, 1981). Page & West (1981) computed the dissociation constant of the unloaded carrier for the proton as about 4×10^{-9} M with a factor of $1 + K_a$, where K_a is the equilibrium constant for the interconversion of the unloaded carrier from the internal to the external surface of the membrane. Indeed, the dissociation constant for lactose or *p*-nitrophenyl α-galactoside at pH 8.0 was twice that at pH 7.6, whereas the maximum number of binding sites was similar at pH 7.6 and pH 8.0 [see Table II of Wright et al. (1981)]. This high affinity of the lactose carrier for the proton may have some physiological implication, because the rate of metabolism is enhanced by a high internal pH (Booth et al., 1979). Thus, it may be necessary to examine the binding affinity exactly in medium greater than pH 8.0.

We do not have direct evidence that glutamate, H^+, and Na^+ bound to the carrier in binding experiments can be translocated across the cytoplasmic membrane when energy is provided. However, the model of glutamate binding (Figure 6) intuitively postulates a model for a syn-coupled transport as presented in the inset of Figure 6. It would be easy to envisage from this model that the Michaelis constant of transport (K_t) depends on the concentrations of both Na^+ and H^+ in the medium.

This model also predicts that both Na^+ and H^+ are coupling ions for the syn-coupled transport reaction, suggesting that a chemical gradient of Na^+ or H^+ can drive glutamate transport. Moreover, because the transfer of a net positive charge occurs along with translocation of glutamate, a membrane potential (interior negative) will enhance the rate of glutamate transport. The following paper gives results indicating that a model such as that shown in the inset of Figure 6 can explain the mechanism of active glutamate transport in *E. coli* B.

Acknowledgments

We are most grateful to Professor K. Aida, University of

Tokyo, for his encouragement and discussion during this work.

Registry No. Glutamic acid, 56-86-0; sodium, 7440-23-5; hydrogen ion, 12408-02-5.

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Mechanism of Glutamate Transport in *Escherichia coli* B. 2. Kinetics of Glutamate Transport Driven by Artificially Imposed Proton and Sodium Ion Gradients across the Cytoplasmic Membrane†

Tsutomu Fujimura, Ichiro Yamato, and Yasuhiro Anraku*

ABSTRACT: Simultaneous imposition of a pH gradient (ΔpH, interior alkaline) and a sodium gradient (ΔpNa, $[Na^+]_{out} > [Na^+]_{in}$) across cytoplasmic membrane vesicles from *Escherichia coli* B led to a several hundred fold accumulation of glutamate. Although less effective, ΔpH (interior alkaline) alone caused accumulation of glutamate in the presence of Na^+. In addition, ΔpNa ($[Na^+]_{out} > [Na^+]_{in}$) alone also drove the transport system, where the maximum level of glutamate accumulation was affected by the pH of the medium. A membrane potential imposed by valinomycin-induced K^+ diffusion (interior negative) enhanced the accumulation, indicating that the system operates in an electrogenic manner. The Michaelis constant of glutamate transport was greatly affected by changes in the concentrations of both Na^+ and H^+ and could be expressed by a linear combination of the reciprocals of the Na^+ and H^+ concentrations in the medium. On the contrary, a membrane potential (interior negative) exerted its effect by increasing the maximum velocity. When membrane vesicles were loaded with glutamate and Na^+, but not with glutamate alone, rapid efflux of glutamate with Na^+ as the cocation down the concentration gradients took place upon dilution. These results indicate that both Na^+ and H^+ are syn-coupled ions of glutamate transport in *E. coli* B and that the carrier/Na^+/H^+/Glu^- complex observed in the binding reaction is an intermediate in the transport.

In the preceding paper (Fujimura et al., 1983), we investigated the binding of glutamate (Glu)[1] to a glutamate carrier in the membrane of *Escherichia coli* B and proposed a binding model (Figure 1): Glutamate can only bind to an Na^+/H^+/carrier complex that is formed by random-order binding of Na^+ and H^+ to the unloaded carrier. Assuming that the

† From the Department of Biology, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan. *Received September 29, 1982.* This research was supported in part by a Grant-in-Aid for a Special Research Project from the Ministry of Education, Science, and Culture of Japan and a grant from the Toray Science Foundation of Japan.

[1] Abbreviations: Glu, glutamate; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; K_d, dissociation constant; K_t, Michaelis constant of transport; V_{max}, maximum velocity of transport; $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of protons; ΔpH, pH gradient; $\Delta\psi$, membrane potential; ΔpNa, chemical gradient of Na^+.

0006-2960/83/0422-1959$01.50/0