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Characterization of a Binding Protein-Dependent Glutamate Transport System of *Rhodobacter sphaeroides*

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The mechanism of L-glutamate uptake was studied in *Rhodobacter sphaeroides*. Uptake of L-glutamate is mediated by a high-affinity ($K_i$ of 1.2 μM), shock-sensitive transport system that is inhibited by vanadate and dependent on the internal pH. From the shock fluid, an L-glutamate-binding protein was isolated and purified. The protein binds L-glutamate (apparent $K_i$ of 1.3 μM) and L-glutamine ($K_i$ of 15 μM) with high affinity. The expression level of this binding protein is maximal at limiting concentrations of glutamine in the growth medium. The glutamate-binding protein restores the uptake of L-glutamate in spheroplasts. L-Aspartate is a strong competitive inhibitor of L-glutamate uptake ($K_i$ of 3 μM) but competes only poorly with L-glutamate for binding to the binding protein ($K_i$ of >200 μM). The uptake of L-aspartate in *R. sphaeroides* also involves a binding protein which is distinct from the L-glutamate-binding protein. These data suggest that in *R. sphaeroides*, the L-glutamate- and L-aspartate-binding proteins interact with the same membrane transporter.

Bacteria may use different types of transport systems to scavenge solutes from the surrounding medium. For example, the binding protein-dependent transport systems are capable of removing their substrates from the environment. These binding protein-dependent transport systems have been found for a variety of substrates, e.g., amino acids, oligopeptides, sugars, and inorganic ions (5, 10, 30). These systems typically consist of five proteins: a soluble or membrane-associated binding protein that binds the substrate with high affinity at the external face of the cytoplasmic membrane, two identical or homologous integral membrane proteins, and two identical or homologous ATP-binding proteins. The ATP-binding proteins are peripherally associated with the cytosolic face of the membrane and couple the hydrolysis of ATP to the transport process (6, 7, 24). In addition to these binding protein-dependent systems, bacteria usually contain secondary transport systems that couple the uptake of solutes to the proton motive force (23).

The purple nonsulfur bacteria (family *Rhodospirillaceae*) are widely distributed in nature and are commonly found in flat, stagnant water bodies in which rapid production and decomposition of organic matter take place. They are capable of changing rapidly their metabolism from an aerobic to an anaerobic mode. A wide range of organic carbon sources can be used to support growth (21).

Thus far, only binding protein-dependent transport systems have been identified in *Rhodobacter* sp. Alanine uptake by *Rhodobacter sphaeroides* is mediated by a shock-sensitive transport system that is inhibited by vanadate (2). Vanadate is considered to be a specific strong inhibitor of P-type ATPases, but it also blocks the uptake of solutes by well-characterized binding protein-dependent transport systems (7, 24). In *R. sphaeroides*, vanadate inhibits the uptake of L-leucine, L-proline, L-glutamine, and L-histidine, suggesting that these amino acids are also accumulated via binding protein-dependent transport systems (1). In *Rhodobacter capsulatus*, a binding protein-dependent transport system has been found for C4 dicarboxylates (26, 27). This extensive use of binding protein-dependent transport systems to accumulate solutes must have a specific physiological advantage, but the nature of this advantage is thus far unknown. To gain more insight in this aspect, we have started to characterize a binding protein-dependent transport system for L-glutamate in *R. sphaeroides*.

The uptake system for L-glutamate was chosen to enable future comparisons with extensively studied secondary glutamate uptake systems (31, 32). The results of this study suggest that *R. sphaeroides* uses distinct binding proteins for the uptake of the anionic amino acids L-glutamate and L-aspartate and that these binding proteins use the same integral membrane components for transport.

**MATERIALS AND METHODS**

**Culture conditions.** *R. sphaeroides* 4P1 (18) was grown aerobically in the dark at 30°C in the medium described by Sistrom (28). Succinate was added as a carbon source, and ammonium chloride was added as a nitrogen source. *R. sphaeroides* 4P1 is a derivative of strain 2.4.1 and harbors a plasmid which contains the *Escherichia coli lacZ* and *lacY* genes and a gene coding for streptomycin resistance under the control of a constitutive *R. sphaeroides* promoter (18). For growth of strain 4P1, streptomycin (50 μg/mL) was added to the medium. Spheroplasts were prepared from cells grown under saturating light conditions up to an optical density at 600 nm of 0.5 to 1. Cells were centrifuged at 10,000 × g for 10 min, and the pellet obtained was resuspended (20 mg/g [wet weight]) in 120 mM potassium phosphate (pH 8.0) containing 20 mM EDTA and 400 μg of lysozyme per mL. After 15 min of incubation at room temperature, the suspension was diluted 1:1 with water. The spheroplast suspension was centrifuged again at 10,000 × g for 10 min, and the pellet was resuspended in 50 mM potassium N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.8) with 5 mM MgSO4 and 50 μg of chloramphenicol per mL.

**Transport assays.** Transport studies in cells were performed as described by Abe et al. (2) at 30°C. Cells were harvested during logarithmic growth, washed twice in the desired buffer, and resuspended to a protein concentration of 5 to 15 mg/mL. Cells were stored on ice until use. For uptake experiments, [14C]lactose and [14C]glutamate were used at final concentrations of 0.2 mM and 9.4 μM, respectively. Vanadate inhibition of transport activity was analyzed as described previously (2) except that uptake studies were performed under aerobic conditions. The substrate specificity of the glutamate uptake system was investigated by measuring the degree of competition of L-glutamate (9.4 μM) uptake by unlabelled substrate analogs (1 mM). Transport of [3H]glutamate by spheroplasts reconstituted with binding protein was essentially done as described above. Purified binding protein was preincubated with [3H]glutamate at room temperature for 5 min prior to the addition of the spheroplasts.

**Isolation of the periplasmic fraction.** For the determination of glutamate...
binding activity in the shock fluid, cells were exposed to the osmotic shock procedure as described by Neu and Heppel (19, 33). The shock fluid was concentrated by ammonium sulfate precipitation to 300 μg/ml and dialyzed against potassium phosphate (pH 7.0). To purify the glutamate-binding protein (GBP), the periplasmic protein fraction was obtained by the procedure described by Ames et al. (6). Cells were harvested during logarithmic growth and resuspended in 50 mM potassium phosphate (pH 7.0) containing 2.5 mM EDTA. Chloroform was added, and the mixture was cautiously shaken for 15 to 30 min and subsequently centrifuged at 10,000 g for 30 min. The supernatant saturated with chloroform was air dried, centrifuged again for 30 min at 10,000 g, and further used for analysis and binding protein purification.

**Purification of the binding protein.** The periplasmic protein solution was loaded on a Mono Q HR 10/10 column connected to a Pharmacia fast protein liquid chromatography (FPLC) system. Elution was at 1 ml/min with a 0.20 to 0.40 M KCl gradient (60 ml) in 50 mM Tris-HCl (pH 7.0). Active fractions were pooled and loaded on a gel filtration column (high-performance Sephadex S-200). Fractions showing a significant level of L-glutamate binding were pooled and loaded on a Mono Q HR 5/5 column with a 0.2 to 0.4 M KCl gradient (60 ml) in Tris-HCl (pH 8.0). Active fractions were pooled. Purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gels according to a standard method (16).

**Binding assay.** Binding of 3H-labelled substrate to the binding protein was measured by the method of Richarme and Kepes (25). The sample (100 μl) was mixed with 1 μl of 3H-labelled glutamate (0.2 μM), and the mixture was incubated by stirring at 30°C for 5 min. Proteins were precipitated by adding 2 ml of an ice-cold saturated ammonium sulfate solution, and the mixture was passed immediately through 0.45-μm-pore-size nitrocellulose filters. Filters were washed with 2 ml of ice-cold 10 mM Tris (pH 7.0) and further processed. The substrate specificity of GBP was investigated by measuring the inhibition of L-glutamate (0.2 μM) binding by a 100-fold excess of unlabelled substrate analogs in the assay mixture.

**Native molecular mass estimations.** A Sephadex S200 column equilibrated in 10 mM Tris (pH 8.0) was used to estimate the molecular mass of the native protein. The column was calibrated with the following proteins: trypsin (24 kDa), myoglobin (17.8 kDa), RNase A (13.7 kDa), and cytochrome c (12.3 kDa).

**Other procedures.** The protein concentration of whole cells and spheroplasts was determined by the method of Lowry et al. (17), using bovine serum albumin as a standard. Protein concentrations of binding protein fractions were determined by the method of Sorensen and Brodbbeck (29). Antibodies against the R. sphaeroides GBP were raised in rabbits as described previously (11). Primary antibodies were detected with a chemiluminescence detection system using the CSPD chemiluminescent substrate as described by the manufacturer (TROPIX, Inc., Bedford, Mass.).

**RESULTS**

Glutamate uptake by R. sphaeroides is shock sensitive and inhibited by vanadate. R. sphaeroides 4P1 cells rapidly accumulate the anionic amino acid L-glutamate. The rate of L-glutamate uptake was dramatically decreased, from 6.2 to 0.8 nmol/min/mg of protein, when the cells were subjected to an osmotic shock (Fig. 1). In contrast, the H+-lactose symport system of E. coli expressed in these cells was hardly affected under these conditions (33.8 and 28.2 nmol/min/mg of protein for nonshocked and shocked cells, respectively). These data are in agreement with previous observations that osmotically shocked cells retain the ability to generate a proton motive force.

To investigate whether the osmotically shocked cells release a periplasmic GBP, binding of L-[14C]glutamate and [14C]lactose by concentrated shock fluid was determined. A high level of L-glutamate binding (12,900 cpm/mg of protein) was detected. There was no significant amount of lactose binding activity. Binding protein-dependent transport systems are usually inhibited by vanadate. Glutamate uptake in R. sphaeroides was found to be strongly inhibited by 1 mM vanadate (Fig. 2B), while hardly any inhibition was observed for lactose uptake (Fig. 2A). It should be noted that in the presence of 1 mM vanadate, the ATP level in the cells is reduced only by 25 to 30% (2). These data suggest that the uptake of L-glutamate by R. sphaeroides cells is mediated by a shock-sensitive, periplasmic binding protein-dependent transport system.

**Isolation of a GBP that reconstitutes L-glutamate transport in spheroplasts.** The GBP present in concentrated shock fluid was purified to homogeneity by Mono Q FPLC and gel filtration. The purified protein migrated on SDS-PAGE as a single band with a molecular mass of 36 kDa (Fig. 3). The molecular
mass estimated by gel filtration was 29 kDa, indicating that the protein is monomeric. The specific L-glutamate binding activity increased 150-fold relative to the concentrated osmotic shock fluid, whereas the final yield of the binding protein was approximately 10%.

The expression level of GBP, as determined by immunoblot analysis, varied with the amount of the nitrogen source supplied for growth. High levels of binding protein were found when ammonium chloride (Fig. 4, lane 1) and glutamine (lane 3) were supplied at limiting concentrations, i.e., 1 and 0.5 mM, respectively. The expression level was reduced three to fivefold when the nitrogen source was supplied in excess, i.e., 40 mM ammonium (lane 2) or 20 mM glutamine (lane 4). Similar results were obtained when the binding of \( ^{14}\text{C}\)glutamate by the periplasmic fraction was measured (data not shown). Glutamate supports growth only poorly when used as a sole nitrogen source. Its effect on the expression level was therefore not investigated.

Spheroplasts accumulate L-glutamate at a low rate. This uptake activity is caused by the presence of whole cells in the preparation and is totally inhibited by the addition of 100 \( \mu \text{M} \) L-aspartate (Fig. 5). However, when the spheroplasts were reconstituted with the purified GBP, uptake of L-glutamate was dramatically increased (Fig. 5). The elevated uptake activity was only slightly inhibited by 100 \( \mu \text{M} \) L-aspartate (data not shown). These data further suggest the mediation of an L-glutamate uptake by a binding protein-dependent transport system.

**Substrate specificity of transport and binding.** The uptake of L-glutamate occurs with a \( K_t \) of 1.2 \( \mu \text{M} \). A 100-fold excess of L-glutamate, L-aspartate, or L-asparagine inhibited L-glutamate uptake by more than 95% (data not shown). L-Aspartate was found to be a competitive inhibitor of L-glutamate uptake, with a \( K_i \) of 3 \( \mu \text{M} \) (Table 1). L-glutamate uptake was also inhibited by a 1,000-fold excess of the L-glutamate analogs \( \beta \)-hydroxyaspartate (100%), glutamate-\( \gamma \)-benzylester (62%), methylglutamate (69%), and \( \delta \)-glutamate (85%). The compounds \( \alpha \)-keto glutarate (34%) and \( \gamma \)-aminobutyrate (30%) had only a slight effect on L-glutamate uptake.

The purified GBP showed a high apparent affinity of \( K_{\text{app}} \) for L-glutamate of 1.3 \( \mu \text{M} \) (Table 1). Scatchard plot analysis of L-glutamate binding revealed a maximal number of sites of 0.7 mol/mol of protein. Glutamate inhibits L-glutamate binding competitively, with a \( K_i \) of 15 \( \mu \text{M} \). In contrast to the inhibition of L-glutamate uptake by L-aspartate and L-asparagine, binding of L-glutamate to the purified binding protein was not inhibited by aspartate and asparagine (\( K_i \) values exceed 200 \( \mu \text{M} \)). However, glutamate binding was inhibited by a 100-fold excess of D-glutamate (57%) and glutamate-\( \gamma \)-benzylester (30%). Hardly any inhibition was observed by \( \gamma \)-aminobutyrate (12%), \( \alpha \)-keto glutarate (20%), and DL-\( \alpha \)-methylglutamate (20%). These data suggest that the purified GBP has affinity only for glutamate and glutamine.

**Glutamate uptake is sensitive to the intracellular pH.** Uptake of glutamate was studied as a function of the intracellular pH. The intracellular pH was varied by resuspending cells in 50 mM potassium phosphate buffer adjusted to different pH values in the presence of 100 mM potassium acetate. Acetate rapidly diffuses into the cell in its protonated form, thereby dissipating the transmembrane pH gradient. This was deduced from the observation that there was no accumulation of \( ^{14}\text{C} \)-benzoic acid in cells incubated in buffers of pH 6.0, 7.0, and 8.0 containing 100 mM potassium acetate. In contrast, in cells without potassium acetate, the accumulation of \( ^{14}\text{C} \)-benzoic acid increased with the increase of \( \Delta \text{pH} \) at decreasing pH (data not shown). The membrane potential remained constant at \(-132 \text{ mV} \) under these conditions (2). Previous studies also demonstrated that the ATP levels in the cell were between 2.0 and 2.2 mM under these conditions (2). In the absence of

**TABLE 1. Kinetic properties of the glutamate transport system and binding protein**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Transport</th>
<th>Binding</th>
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</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Glutamine</td>
<td>ND*</td>
<td>15</td>
</tr>
<tr>
<td>Aspartate</td>
<td>3</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Asparagine</td>
<td>ND</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

* \( K_i \) values were estimated from the inhibition of L-glutamate uptake by whole cells or L-[\( ^{3}\text{H} \)]glutamate binding to the purified GBP. For binding, apparent \( K_i \) and \( K_i \) values are shown.

<sup>a</sup> ND, not determined.

**FIG. 5.** (A) Uptake of \( ^{3}\text{H} \) glutamate in spheroplasts with (C) and without (V) GBP (0.1 \( \mu \text{g} \)) and in the absence (open symbols) or presence (closed symbols) of 100 \( \mu \text{M} \) aspartate. (B) Stimulation of glutamate uptake by GBP with (C) and without (D) 100 \( \mu \text{M} \) aspartate.
7.0, suggesting that its pl is below 7.0. Polyclonal antibodies raised against the purified GBP of *R. sphaeroides* do not react with a cell extract of *E. coli* (13), while the glutamate/aspartate-binding proteins of *E. coli* and *S. typhimurium* were found to be cross-reactive (15). The gram-positiveter bacterium *Lactococcus lactis* also has a high-affinity glutamate/glutamine transport system (22) that functions most likely as an ATP- and binding protein-dependent transport system. The binding protein of this bacterium may thus resemble the GBP described in this report, at least with respect to its specificity. An immunoblot with a cell extract of *L. lactis* incubated with the polyclonal antibodies raised against GBP showed a weak band (13). The molecular mass of the *R. sphaeroides* GBP as determined by SDS-PAGE is 36 kDa, a value that fits in the range of the sizes reported for a variety of other periplasmic binding proteins (30).

The purified GBP has no affinity for aspartate. Aspartate, however, efficiently reduces the uptake of glutamate in a competitive manner, but it inhibits only slightly the GBP-stimulated glutamate transport in spheroplasts. Since aspartate transport is also osmotic shock sensitive, it is likely that *R. sphaeroides* contains a separate periplasmic binding protein that is involved in the high-affinity uptake of aspartate. A low L-aspartate binding activity was found in the concentrated shock fluid, but so far, we have been unable to purify this aspartate-binding protein. This aspartate-binding protein appears to interact with the same integral membrane complex that mediates the uptake of glutamate. Recently, mutants of *R. sphaeroides* that are deficient in the uptake of glutamate and aspartate but still express the GBP were obtained (13). These mutants will allow the cloning of the genetic determinants that define glutamate and aspartate uptake. The existence of two binding proteins that interact with the same membrane complex has been reported before. Histidine and arginine transport in *S. typhimurium* (12) involves at least two binding proteins: HisJ, which is specific for histidine only, and LAO, with specificity for lysine, arginine, and ornithine. HisJ and LAO associate with the membrane components HisQ and M. Another example of two binding proteins using the same membrane complex is the leucine-specific binding protein and the leucine-, isoleucine-, and valine-binding protein (3).

Many bacteria contain, in addition to a binding protein-dependent system, one or more protons motive force-driven transport systems for a set of substrates. Proton motive force-driven systems presumably require less metabolic energy per solute transported (one-third to one-half ATP equivalent) than the binding protein-dependent systems (one ATP) (14). *R. sphaeroides* appears to lack such proton motive force-dependent systems and seems to rely solely on binding protein-dependent transport systems. This implies that the presence of such energy-demanding high-affinity but usually low-capacity transport systems provides this organism with a specific physiological advantage. Under anaerobic-light conditions when the energy supply is in excess, *R. sphaeroides* can afford to spend so much energy for the uptake of nutrients. However, under aerobic-dark conditions, the metabolic energy supply is not always in excess. Furthermore, *R. sphaeroides* also uses binding protein- and ATP-dependent systems for nutrients that it can synthesize, like amino acids. A more likely explanation for the use of only binding protein-dependent systems for the uptake of nutrients is that these systems are irreversible and that during energy-limited conditions, loss of intracellular nutrients into the environment does not occur via the transport systems.

The activity of the glutamate transport system varies with the intracellular pH, with a pK of 7.2. A higher pK value, i.e., 7.8,
was found for alanine and malate uptake in *R. sphaeroides* and *R. capsulatus*, respectively (2, 27). Under anaerobic-dark conditions, the intracellular pH drops rapidly to below pH 7.8. As a result, the transport activities decrease drastically and no ATP is consumed by transport processes. In this way, the cells maintain a high ATP content for maintenance purposes. Solutes are not lost under these conditions since the binding proteins-dependent transport systems are unidirectional. Under more favorable conditions, i.e., anaerobic-light conditions, the intracellular pH increases again and ATP can be used for the energization of transport. This hypothesis can now be tested by transport studies with the available transport mutants.

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