Purification and Characterization of a Histidine-binding Protein from Salmonella typhimurium LT-2 and Its Relationship to the Histidine Permease System*

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SUMMARY

A histidine-binding protein has been isolated from the shock fluid of osmotically shocked Salmonella typhimurium cells and has been purified by conventional techniques. It has a molecular weight of 25,000 and binds 1 histidine molecule per molecule of protein. The binding protein is stable to wide variations of temperature, ionic strength, and pH. It binds histidine with a $K_D$ of 1.5 µM. The protein also binds arginine but with a much weaker affinity.

The transport of histidine by Salmonella typhimurium was found to be an energy dependent process. The $K_m$ for histidine was 0.07 µM when measured by the "growing cells" method but it was 1 µM when determined in the presence of an inhibitor of protein synthesis. Histidine transport is inhibited by large excesses of arginine. Osmotically shocked cells exhibit a reduction in the initial rate and the steady state level of histidine accumulation.

The simultaneous release of the histidine-binding protein and the reduction of transport caused by osmotic shock, as well as the similarity of kinetic constants and specificity of the binding protein and the histidine permease system suggest that the histidine-binding protein is a functional component of the histidine permease system.

* This work was supported by Grant GM-12717 from the United States Public Health Service.
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cultures were obtained by inoculating S. typhimurium LT-2 into a bubbler tube containing 10 to 20 ml of the E medium of Vogel and Bonner (12) supplemented with 0.5% glucose and 1 ml of trace elements per liter of medium and aerating continuously at 37° for approximately 15 hours. Each milliliter of trace elements contained CoSO₄ 2.6 µg; H₃BO₃ 5.7 µg; (NH₄)₂MoO₄·4H₂O, 15 µg; FeCl₃ 483 µg; MnCl₂·4H₂O, 278 µg; CuCl₂ 269 µg; and ZnCl₂ 2.08 µg. One aliquot of the overnight culture was subinoculated into 100 or 300 ml of fresh growth medium in an Ehrlenmyer flask, allowed to grow with continuous shaking on a New Brunswick gyrotory shaker, and harvested when the absorbance at 650 nm reached a value of 0.5 to 0.9 on a Zeiss PMQII spectrophotometer, corresponding to approximately 5 to 9 × 10⁸ cells per ml or midexponential phase. For the last batches used in the purification experiments 300 ml of a stationary phase culture of S. typhimurium LT-2 cells were subinoculated into 5-gallon carboys containing 15 liters of fresh growth medium and allowed to grow with vigorous aeration at 37°; aeration was provided through a sintered glass sparger. The medium and allowed to grow with vigorous aeration at 37°. Ehrlenmyer flask, allowed to grow with continuous shaking on a New Brunswick gyrotory shaker, and harvested when the absorbance at 600 nm reached a value of 0.5 to 0.9 on a Zeiss PMQII spectrophotometer, corresponding to approximately 5 to 9 × 10⁸ cells per ml or midexponential phase.

The cell suspension was stirred with a magnetic stirrer for 10 min at room temperature and was then centrifuged at 16,000 × g for 20 min. The cell suspension was then centrifuged and resuspended in 40 volumes of room temperature 0.033 M Tris-HCl, pH 7.2. An equal volume of the same buffer containing 40% sucrose was added, followed rapidly by 5 ml per liter of 0.02 M EDTA to form a final concentration of 0.1 m EDTA. The cell suspension was stirred with a magnetic stirrer for 10 min at room temperature and then centrifuged at 16,000 × g for 20 min. The washed cells were centrifuged and resuspended in 40 volumes of room temperature 0.033 M Tris-HCl, pH 7.2. An equal volume of the same buffer containing 40% sucrose was added, followed rapidly by 5 ml per liter of 0.02 M EDTA to form a final concentration of 0.1 m EDTA. The cell suspension was then centrifuged for 20 min at 16,000 × g. The supernatant solution or "shock fluid" containing the histidine-binding protein was carefully decanted.

**Assay for Binding Activity**—Binding activity was determined as follows: 250 to 300 µl of either crude shock fluid (0.5 to 4 mg of protein per ml) or a solution of the purified binding protein (0.03 to 0.1 mg of protein per ml) was pipetted into a 1-inch diameter dialysis bag tied at both ends and dialyzed for at least 10 hours at 4° against 5 ml of 0.05 m sodium phosphate buffer, pH 6.5, containing the labeled substrate. For most assays 1 µM L-[¹⁴C]-histidine was used. The binding activity was nearly linear within these protein ranges. When multiple assays were performed against the same concentration of substrate, each dialysis bag was labeled and placed into a beaker containing 5 ml of substrate solution per bag. The dialysis solutions were stirred constantly with a magnetic stirrer. A trace of chloroform was added to the assay solutions to prevent bacterial growth.

At the end of the dialysis, each bag was removed from the assay medium and pierced with a 50-µl Hamilton syringe equipped with a Chaney adaptor to remove one aliquot of the binding solution. Two 50-µl samples were spotted on a 2.3-cm Whatman No. 3MM filter disc, dried either by air or in an oven at 85° for 15 min, and placed into 5 ml of Toluene-Liquidfluor (New England Nuclear), and the radioactivity was measured for 10 min in either a Tri-Carb liquid scintillation spectrometer model 338-544 (Packard Instrument Company) or in a Beckman LS-100 scintillation counter (Beckman Instruments). The radioactivity in samples of the outside medium (dialysate) was measured both before and after dialysis. Binding capacity was calculated according to the method of Peurose et al. (10):

\[ P_0 = \frac{(\text{sample counts per min} - \text{dialysate counts per min})}{(K_D + S)} \]

where \( P_0 \) is the amount of substrate bound at saturating substrate concentration, \( K_D \) is the dissociation constant of the protein-substrate complex, and \( S \) is the final substrate concentration. All values are in micromolar units, and 1 unit of binding activity is defined as 1 nmole of binding site. For histidine an average value of 1.5 µM was determined for the \( K_D \) of the histidine-protein complex. The value for \( P_0 \) was found to be negligible when bovine serum albumin was used in place of the histidine-binding protein.

**Assay for Histidine Transport**—Cells were harvested in logarithmic phase, as described above, washed twice with 40 volumes of 0.01 M Tris-HCl:0.03 M NaCl, pH 7.3. The cells were then suspended to a concentration of 5 × 10⁸ cells per ml as measured spectrophotometrically in E-C medium, as described by Ames (14). E-C medium is essentially Medium E of Vogel and Bonner (12), except that citrate is omitted, and the medium is adjusted to pH 7.3 with HCl. One milliliter of transport assay medium contained 0.2 ml of the cell suspension and 40 µg per ml of chloramphenicol. Other substances were added as indicated. The assay medium was brought to 1 ml by the addition of E-C medium. The histidine level varied depending on the nature of the experiment, but, for most assays, 1 µM histidine was used.

Up to 5 ml of the assay medium were placed in a 25-ml Ehrlenmyer flask and shaken without substrate for either 10 or 90 min at 25° in a Duhnhoff metabolic incubator. Substrate was then added and shaking was continued. Aliquots of 0.4 ml were withdrawn at designated time periods, filtered through Millipore HA membranes (25 mm, 0.45 µm pore size) with a Millipore sampling manifold. The filters were washed twice with 2 ml each of an ice-cold solution of the washing buffer of Anraku (18), consisting of 0.01 M Tris-HCl:0.5 mm MgCl₂:0.15 M NaCl, pH 7.5. The filters were then dried for 15 min at 85°, placed in 5 ml of Toluene-Liquidfluor, and the radioactivity was measured in a liquid scintillation counter for 10 min. Results are expressed as nanomoles of L-histidine taken up per 10⁶ cells.

Accumulation of unmetabolized histidine by the cells was determined as follows. A 0.5-ml aliquot of the incubation suspension was removed 2 min after the addition of labeled histidine, filtered through a Millipore filter as described above, washed, and extracted overnight with 2.5 ml of cold 75% ethanol. One al-
The solution was dialyzed overnight against the same buffer. Following dialysis, the solution contained 355 mg of protein in 195 ml. Five milliliters were set aside for assay purposes. All purification steps were performed at room temperature, except the dialyses which were performed at 4°C.

### Results

**Concentration of Osmotic Shock Fluid**—Two hundred fifty grams (wet weight) of midlogarithmic phase *S. typhimurium* LT-2, representing the yield from 92 liters of medium, were subjected to cold osmotic shock procedure as described under "Materials and Methods." The shock fluid was concentrated to 2 liters by ultrafiltration at 95 p.s.i. of nitrogen gas at 4°C in a model 410 ultrafiltration cell equipped with a PM-10 membrane. To concentrate the shock fluid further, the solution was made 95% saturated with respect to ammonium sulfate and stirred for 3 hours at room temperature. The precipitated protein was recovered by centrifugation at 16,000 × g for 20 min, redissolved in 0.05 M sodium phosphate buffer, pH 6.5, and dialyzed exhaustively against the same buffer. Following dialysis, the solution contained 355 mg of protein in 195 ml. Five milliliters were set aside for assay purposes. All purification steps were performed at room temperature, except the dialyses which were performed at 4°C.

### Table I

**Purification of histidine-binding protein from Salmonella typhimurium LT-2**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Binding units</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Concentrated shock fluid</td>
<td>190</td>
<td>655</td>
<td>246</td>
<td>1.90</td>
<td>100</td>
</tr>
<tr>
<td>2. Ammonium sulfate...</td>
<td>20</td>
<td>519</td>
<td>203</td>
<td>2.53</td>
<td>78</td>
</tr>
<tr>
<td>3. Carboxymethyl-cellulose...</td>
<td>38</td>
<td>342</td>
<td>49</td>
<td>6.93</td>
<td>52</td>
</tr>
<tr>
<td>4. Hydroxylapatite...</td>
<td>4.5</td>
<td>110</td>
<td>3</td>
<td>35.80</td>
<td>17</td>
</tr>
</tbody>
</table>

The purification procedure is given in the text. One unit of binding activity is equivalent to 1 nmole of binding site.
Histidine-binding Protein

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Fig. 1. Purification of the histidine- and the isoleucine-binding proteins by carboxymethylcellulose column chromatography. Details of the purification procedure are given in the text. Arrow 1 indicates the point where elution with a linear gradient of sodium acetate and NaCl was begun. Arrow 2 indicates the point at which elution with 1 M NaCl was begun. O-O, absorbance; O---O, histidine-binding activity in counts per min; W---W, isoleucine-binding activity in counts per min.

Fig. 2. Purification of the histidine-binding protein by hydroxylapatite column chromatography. The details of the purification are given in the text. A stepwise elution with increasing concentrations of potassium phosphate buffer, pH 6.5, was performed. Arrows 1 through 9 indicate in increasing order of molarity the points in the elution where the buffers used in the stepwise elution were applied. Arrows 1 through 9 represent 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, and 0.02 M potassium phosphate buffer, pH 6.5, respectively. O---O, absorbance; O----O, histidine-binding activity.

Hydroxylapatite Chromatography—Commercial hydroxylapatite was poured into a 2.5-cm column to a packed height of 9.5 cm and was equilibrated with 8 volumes of 0.001 M potassium phosphate buffer, pH 6.5. The dialyzed protein was applied to the column in 38 ml and washed with 1.5 column volumes of buffer. Next, a stepwise elution with potassium phosphate buffers at different concentrations was performed under slight pressure applied with a Davol cautery set, with 1.5 column volumes (70 ml) for each step. The concentrations of phosphate buffers used in the stepwise elution were 0.05, 0.06, 0.07, 0.08,

Fig. 3. Continuous polyacrylamide gel electrophoresis. Eight-millimeter diameter tubes were filled to 7 cm with a 6% acrylamide solution in 0.05 M sodium acetate buffer, pH 5.0. Protein was layered on the top of the gels and electrophoresed for 10 min at 2.5 ma per tube followed by 150 min at 5 ma per tube. The direction of migration was toward the cathode, corresponding to the bottom of the figure. 1, 140 µg of the redissolved and dialyzed 60 to 95% ammonium sulfate precipitate; 2, 27 µg of the purified histidine-binding protein.

0.09, 0.10, 0.11, 0.12, and 0.20 M, each adjusted to pH 6.5. Seven-milliliter fractions were collected, and the absorbance for each fraction was subsequently determined at 230 nm. Fractions 10 through 33 contained histidine-binding activity (Fig. 2). Fractions 23 through 30 were pooled and concentrated to 4.5 ml by ultrafiltration. The purification of the histidine-binding protein is summarized in Table I.

Criteria for Purity—The histidine-binding protein was judged to be nearly homogeneous by disc gel electrophoresis, sedimentation velocity, and equilibrium ultracentrifugation.

Polyacrylamide Disc Gel Electrophoresis—Polyacrylamide disc gel electrophoresis was performed under three different sets of conditions, with the use of a basic buffer system, an acidic buffer system, and an acidic buffer system incorporating discontinuous buffers. The first set of gels was run in 0.05 M Tris-acetate buffer, pH 9.2, in which the direction of migration was toward the anode. In each of these two sets of gels, 27 µg of purified protein were applied to the surface of a 6% gel. In each case, a single sharp band resulted.

A third set of gels was run with the discontinuous system of Reisfeld, Lewis, and Williams (18) in which a buffer consisting of β-alanine-acetic acid, pH 3.8, was run through a 7.5% gel at pH 4.3. The direction of migration was toward the cathode. Samples of 70 and 140 µl were used, and one faint band was detected above a very heavy major band.
Sedimentation Velocity Ultracentrifugation—Sedimentation velocity ultracentrifugation was performed with a Spinco model E analytical ultracentrifuge, equipped with a scanner. Trajectories were taken every 16 min. Only one plateau region was observed. A plot of ln r against time (Fig. 4), where r is the distance of the midpoint of the gradient from the center of rotation. The slope of the line, d ln r/dt, is proportional to a sedimentation coefficient of 2.6 S.

Sedimentation Equilibrium Ultracentrifugation—The molecular weight of the purified binding protein was determined by high speed equilibrium ultracentrifugation in a Spinco model E analytical ultracentrifuge, according to the method of Yphantis (20). An external loading multichannel cell, 12-mm long, was filled to a 3-mm column height, with a different concentration of the histidine-binding protein in each channel. The cell was placed in an AN-D rotor and centrifuged at 40,000 rpm in a Spinco model E ultracentrifuge at 20°C. The absorbance at 288 nm was measured by absorption optics and printed by a scanner at intervals of 16 min. r is the distance of the midpoint of the gradient from the center of rotation. The slope of the line, d ln r/dt, is proportional to a sedimentation coefficient of 2.6 S.

Fig. 4. Sedimentation velocity ultracentrifugation of the purified histidine-binding protein. Histidine-binding protein (50 μg per ml) in 0.06 M potassium phosphate buffer, pH 6.5, was placed in one sector of a 12-mm double sector cell with the same phosphate buffer in the other sector and was centrifuged at 50,000 rpm in a Spinco model E ultracentrifuge at 20°C. The absorbance at 288 nm was measured by absorption optics and printed by a scanner at intervals of 16 min. r is the distance of the midpoint of the gradient from the center of rotation. The slope of the line, d ln r/dt, is proportional to a sedimentation coefficient of 2.6 S.

Fig. 5. High speed sedimentation equilibrium ultracentrifugation of the purified histidine-binding protein. Details are given in the text. The reference channels were filled with 0.06 M potassium phosphate buffer, pH 6.5. The histidine-binding protein was exhaustively dialyzed against and diluted with the same phosphate buffer used for filling the reference channels. The concentrations of histidine-binding protein used were: •, 0.10 mg per ml; □, 0.30 mg per ml; and ○, 0.60 mg per ml.

Fig. 6. Binding as a function of histidine concentration. All assays were performed as described under “Materials and Methods” with 50 μg per ml of partially purified histidine binding protein.

Effects of Temperature and Storage on Histidine-binding Protein—To determine the effect of temperature on the activity of the histidine-binding protein, 1-ml aliquots of the crude binding protein were heated to 95°C in a water bath, cooled rapidly to 4°C, and centrifuged at 35,000 χ g to remove precipitated protein. There was little loss of binding activity after as long as 60 min of heating at 95°C. Binding activity was measured by equilibrium dialysis.

The assay for histidine-binding activity was routinely performed at 4°C. The histidine-binding protein was normally stored at −25°C by quickly freezing 0.5-ml aliquots in a Dry Ice-acetone mixture. Once thawed, the binding protein was kept at 4°C. Little loss of activity was noted upon storage at −25°C for several months.

Effects of pH on Histidine Binding—The binding of histidine by the histidine-binding protein was determined at a series of pH values: from pH 5 to 5.5 in 0.05 M sodium acetate buffer; pH 6 to 8 in 0.05 M sodium phosphate buffer; and pH 8.5 to 10 in 0.05 M sodium borate buffer. There was no effect of pH between 5 and 9, with a gradual loss of activity to 70% of the maximal (pH 6.5 activity) at pH 10. The protein could also be precipitated with...
12% ice-cold perchloric acid and immediately redissolved and adjusted to pH 6.5 with 6 N KOH with no loss of activity.

Specificity of Histidine-binding Protein—The specificity of the binding reaction was determined by competition studies with other potential substrates. The inhibition of L-[14C]histidine binding by the naturally occurring amino acids was determined. Each amino acid was tested at a 200:1 ratio (0.2 mM unlabeled competitor to 1 mM L-[14C]histidine). Rather than using each amino acid separately, they were subdivided into five major categories: the aromatic, sulfur, dicarboxylic acid (and their amides), neutral, and basic amino acids. The results are shown in Table II.

Only the sulfur and basic amino acids caused any significant reduction in the histidine-binding activity of the histidine-binding protein. An amino acid analysis of these two groups of amino acids showed no contamination with histidine. However, the sulfur amino acids were found to be contaminated with a high concentration of basic amino acids which may account for the observed inhibition by the sulfur amino acids used in this study.

Preliminary experiments with the basic amino acids show that lysine alone neither binds to the histidine-binding protein nor inhibits the binding of histidine to any significant extent. Arginine inhibits histidine binding by 10 to 20% and is bound by the purified binding protein approximately 10% as much as histidine when both amino acids are present at 1 mM in the assay medium. Crude shock fluid binds 3 times as much arginine as histidine. This result indicates that in S. typhimurium the total arginine-binding activity is not accounted for by the histidine-binding protein. This is not surprising, since E. coli has been shown to contain a number of basic amino acid-binding proteins (5).

Possible Enzyme Activity of Histidine-binding Protein—Since enzymes which have histidine as their substrate might be expected to bind histidine, the purified protein was assayed for histidyl-tRNA synthetase activity. This enzyme activity was measured according to a modification of the procedure of Munch and Berg (16). After 10, 20, and 30 min of incubation, the protein showed no significant difference from a water blank in histidyl-tRNA synthetase activity. A whole cell sonicate of S. typhimurium, on the other hand, showed linear activity during the same time interval. This result indicates that the histidine-binding protein and histidyl-tRNA synthetase are separate proteins.

S. typhimurium LT-2 has no degradative pathway for histidine; histidine appears to be used only for protein synthesis in this organism (14). For this reason, no other enzymatic activities were measured.

Histidine Transport in S. typhimurium LT-2—The accumulation of histidine by the histidine permease system of S. typhimurium LT-2 was found to be an energy-dependent process, in confirmation of Ames (14). The steady state level of histidine accumulation is decreased by the metabolic inhibitors potassium cyanide, 2,4-dinitrophenol, and sodium azide (Table III, Experiments 1 and 2). When the prior incubation and assay were performed at 0 to 3°C in an ice bath, only 0.1 nmole of histidine per 10¹⁰ cells was taken up, compared to 3.1 nmoles per 10¹⁰ cells for cells assayed at 25°C (Table III, Experiment 3). Moreover, this value at 0–3°C is independent of time, remaining constant from 30 sec to 30 min, the longest time period measured. This may indicate a rapid equilibration of external and internal pool histidine with no net accumulation, i.e., facilitated diffusion.

The possibility of adsorption of histidine to the cells was also investigated. Cells were placed in a boiling water bath for 15 min prior to the beginning of the assay (Table III, Experiment 3). The retention of labeled histidine by the boiled cells was time-independent, amounting to 0.06 nmole per 10¹⁰ cells or 2% of the steady state level of control cells. This result may indicate either nonspecific adsorption of histidine to cellular material or specific binding by the histidine-binding protein which is heat stable. In subsequent experiments, all uptake values were corrected for adsorption by subtracting the amount of histidine re-

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**Table II**

<table>
<thead>
<tr>
<th>Amino acid group</th>
<th>Percentage of inhibition of histidine binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatic</td>
<td>0</td>
</tr>
<tr>
<td>Sulfur</td>
<td>18</td>
</tr>
<tr>
<td>Dicarboxylic</td>
<td>5</td>
</tr>
<tr>
<td>Neutral</td>
<td>0</td>
</tr>
<tr>
<td>Basic</td>
<td>21</td>
</tr>
</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Histidine taken up (nmoles/10¹⁰ cells)</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.88</td>
<td>11</td>
</tr>
<tr>
<td>Control + DNP (0.17 mM)</td>
<td>1.32</td>
<td>41</td>
</tr>
<tr>
<td>Control + DNP (0.5 mM)</td>
<td>5.78</td>
<td>33</td>
</tr>
<tr>
<td>Control + NaNO₃ (30 mM)</td>
<td>0.77</td>
<td>13</td>
</tr>
<tr>
<td>Experiment 3: Effect of low temperature, boiling</td>
<td>Control</td>
<td>3.10</td>
</tr>
<tr>
<td></td>
<td>Control, 0°C</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Control, boiled 15 min</td>
<td>0.06</td>
</tr>
</tbody>
</table>

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port of approximately 2.5 to 5.0. Preliminary experiments indicate a Kᵢ of arginine for histidine transport simultaneously with labeled histidine at a 200:1 ratio (0.2 mM). In these experiments, unlabeled arginine was added after the addition of labeled histidine. The effects of the sulfur-containing amino acids were not studied further as contaminants in the sulfur amino acid preparations. The amino acids which, as revealed by amino acid analysis, were present included histidine (Fig. 7B). The inhibitory action of the sulfur-containing amino acids, methionine and cysteine, and the basic amino acids, arginine and lysine, caused a significant loss of uptake values of boiled cells were significant in initial rate experiments.

Specificity of Histidine Permease—The naturally occurring amino acids were divided into five groups and assayed for their ability to cause the countertransport of labeled histidine from previously loaded cells by the procedure described in the legend to Fig. 7. The aromatic, neutral, and dicarboxylic acid group: tryptophan, tyrosine, phenylalanine; c, dicarboxylic acid group: aspartic acid, asparagine, glutamic acid, glutamine; d, neutral group: alanine, valine, threonine, leucine, isoleucine; e, sulfur group: cysteine, methionine; f, basic group: arginine, lysine; g, unlabeled histidine. The final concentration of each amino acid was 0.2 mM. The assays were performed as described under "Materials and Methods.''

The competition exhibited by arginine was investigated further. In these experiments, unlabeled arginine was added simultaneously with labeled histidine at a 200:1 ratio (0.2 mM arginine to 1 mM [14C]histidine, final concentration). Addition of arginine consistently resulted in a 70% inhibition of histidine accumulation after 2 min. Arginine analysis of our arginine preparations did not detect the presence of any histidine and, therefore, an actual competition by arginine is indicated. Preliminary experiments indicate a Kᵢ of arginine for histidine transport of approximately 2.5 to 5.0 × 10⁻⁴ M.

Glucose affected both the initial rate and the total accumulation of histidine (Fig. 8). The presence of 28 mM glucose during both the previous incubation period and the assay period caused an increase in the initial rate of histidine uptake by control cells, sometimes as much as 100%. However, glucose always caused a decrease in the total accumulation of histidine. Glucose had the same effect on histidine uptake by shocked cells but it usually increased the initial rate in control cells to a greater extent than in shocked cells. Thus, the difference in the histidine-transport capabilities of control cells and of shocked cells was usually accentuated in the presence of glucose.

Kinetics of Histidine Transport—The affinity of the transport system for histidine is shown in Fig. 9. In confirmation of the findings of Ames (14), both a high and a low affinity system for histidine transport were observed. However, the Kᵢ of the high affinity system was dependent upon the experimental conditions used to measure it. When the growing cells method of Ames (14) was used, the Kᵢ of the high affinity system was about 7 × 10⁻⁴ M (Fig. 9B), a value similar to the Kᵢ of 8 × 10⁻⁴ M reported by Ames (14) and Ames and Roth (21) and of 3 × 10⁻⁴ M reported
FIG. 9. Kinetics of histidine transport. A, $K_m$ was determined in the presence of chloramphenicol (40 $\mu$g per ml) with cells which had been starved for 10 min. Initial rates were approximated by 30-s values. B, $K_m$ was determined by the "growing cells" method. Initial rates were approximated by 1-min values. The assays were performed as described under "Materials and Methods."

more recently by Ames and Lever (9). In the growing cells method, histidine uptake is determined by measuring the incorporation of histidine into cell proteins, utilizing histidine concentrations ($1 \times 10^{-8}$ M to $1 \times 10^{-7}$ M) which are believed to make the transport of histidine the rate-limiting step.

When the 90-min starvation method of Ames (14) was used, $K_m$ values of $3 \times 10^{-7}$ M and $1 \times 10^{-6}$ M were obtained for the high affinity and the low affinity systems, respectively, in good agreement with Ames (14). In this method, cells are starved for 90 min in the absence of a carbon source and an inhibitor of protein synthesis. According to Ames (14), under these conditions protein synthesis does not occur in the assay medium used to measure histidine transport. We did not find this to be the case. In our experiments, cells that were starved for 90 min synthesized protein at 60% to 70% of the rate observed in cells which had not been starved. Therefore, to ensure the measurement of histidine transport in a test system that was not complicated by the simultaneous occurrence of protein synthesis, we routinely included chloramphenicol (40 $\mu$g per ml), except when using the growing cell method (14), in the assay medium used to measure histidine transport. In the presence of chloramphenicol, both a high and a low affinity system for histidine transport were still observed (Fig. 9A). The $K_m$ ($1 \times 10^{-4}$ M) of the low affinity system was unchanged in the presence of chloramphenicol but the $K_m$ of the high affinity system was increased to $1 \times 10^{-6}$ M. The same results were obtained when chlorotetracycline was used to inhibit protein synthesis. Chloramphenicol and chlorotetracycline inhibited the initial rate of histidine uptake approximately 20% to 30%.

DISCUSSION

In confirmation of the work of Ames (14), we have found that histidine is accumulated in an active transport process by both a high and a low affinity transport system in S. typhimurium LT-2. Histidine is transported in an unaltered form by a process that concentrates histidine against a gradient, obeys saturation kinetics, and requires a functional respiratory chain, as shown by the inhibitory effects of 2,4-dinitrophenol, potassium cyanide, and sodium azide.

S. typhimurium has both a high affinity and a low affinity system for histidine transport. The $K_m$ of the high affinity system was found to be dependent upon the experimental conditions used to measure it. By the growing cells method and the 90-min starvation method $K_m$ values of $7 \times 10^{-8}$ M and $3 \times 10^{-7}$ M, respectively, were obtained, in good agreement with Ames (14). In these methods, no inhibitor of protein synthesis is added to the test system. When measured in the presence of either chloramphenicol or chlorotetracycline, inhibitors of protein synthesis, the $K_m$ of the high affinity system was $1 \times 10^{-6}$ M.

The initial rate and extent of histidine transport are dependent upon the energy state of the cell. Cells which are allowed to deplete their energy supply by starvation for 90 min exhibit a significant decrease in the initial rate of histidine transport as compared to cells starved for only 10 min. The addition of glucose as an energy source increased the initial rate of transport of histidine, but it decreased its net accumulation, in agreement with the observation of Ames (14). Glucose had the same effect on histidine transport in osmotically shocked cells.

Both the initial rate of histidine transport and the net accumulation of histidine are reduced in S. typhimurium cells subjected to the osmotic shock procedure of Nossal and Heppel (13). Similar findings following osmotic shock have been observed for the transport of a number of other amino acids (3-8). The osmotic shock procedure most likely causes damage to or the release of some substance essential to the transport process. It is known that the function of cellular organelles is impaired by osmotic shock (22); a similar impairment of the energy-dependent pump or a decreased availability of the pump substrate may account for the decreased transport capability of shocked cells. The identity of the pump substrate is not known yet. Recently Klein,
P-galactosides.

membrane state generated during oxidative phosphorylation.

Dahms, and Boyer (23) suggested that the active transport of amino acids may involve utilization of a high energy compound or membrane state generated during oxidative phosphorylation. Since it has been shown that the accumulation of sugars also is reduced by osmotic shock (5), the possibility exists that a reduced rate of glucose transport in shocked cells limits the availability of the pump substrate in these cells. This possibility does not appear likely, however, because the differences in transport ability between control and shocked cells was not eliminated by previous incubation of shocked cells for 10 min in the presence of glucose, a period of time that should have been adequate for the uptake of significant amounts of glucose. Moreover, the fact that glucose alters the initial rate of histidine transport and the total accumulation of histidine in shocked cells in the same manner as it does in normal cells indicates that under our experimental conditions glucose entered shocked cells in sufficient quantity to exert its physiological effects.

These observations suggest, then, that the osmotic shock procedure causes the release from the cells of an essential component of the histidine-transport system. Concomitant with the decrease in the transport of histidine by cells subjected to osmotic shock is the release into the shock fluid of a binding protein which is highly specific for histidine. Of the amino acids tested, arginine is the only one which inhibited histidine binding to any significant extent and this occurred in the presence of an arginine to histidine ratio of 200:1. At this concentration ratio, arginine inhibited histidine binding only 10% to 20%. Arginine is bound by the binding protein but only slightly. When both histidine and arginine were present in the assay medium in equal molar amounts (1 mM), only 10% as much arginine was bound as histidine. Arginine also is the only amino acid which significantly inhibited histidine uptake by intact cells.

The significant amount of transport activity still remaining in shocked cells is most likely due to the release of only a portion of the total binding protein within the cell. Preliminary experiments revealed that, when cells were osmotically shocked a second and a third time, additional binding protein was released and a further reduction in the cell’s ability to transport histidine occurred. However, a significant decrease in the number of viable cells also occurred. After the first osmotic shocking procedure, 100% viability remained, but the second and third treatments killed approximately 50% of the cells.

The binding protein is stable to heating at 90° for 60 min, and its activity is not significantly affected by wide variations in ionic strength and pH. The purified histidine-binding protein has a molecular weight of 25,000 and binds 36 nmoles of histidine per mg of protein. Since 1 mg of the binding protein represents 40 nmoles of the protein, 0.9 mole of histidine is bound per mole of binding protein. This finding suggests that the histidine-binding protein has one binding site for histidine.

While the nature of the bond between the binding protein and histidine has not been determined, there is evidence ruling out a strictly ionic type of binding. First, the ionic strength of the assay medium has no consistent effect on the binding reaction. Second, the charge on the histidine molecule does not appear to be an important factor in the formation of the protein-ligand complex. Histidine has three possible charged groups: the carboxyl group with a pK of 1.8, the imidazole group with a pK of 6.0, and the α-amino group with a pK of 9.2. It was not possible to examine the effects of variations in pH below the pK of the carboxyl group because of reversible precipitation of the protein. However, at pH 5, both the imidazole and α-amino groups are, for all practical purposes, fully protonated. At this pH the binding reaction is unaffected when compared to the binding activity at pH 6.5. At the other extreme, pH 9.9, the imidazole group is uncharged and 80% of the α-amino group is uncharged. Yet at this pH, the histidine binding protein retains 70% of its maximal activity. Only the extent of binding under equilibrium conditions was measured, so that it is possible that the rate of binding was affected by pH or ionic strength. Since this would require an equal change in both the forward and reverse rate constant, however, such effects are unlikely.

Attempts at restoration of full transport activity in shocked cells by the addition of the crude shock fluid and the purified histidine-binding protein to shocked cells have not been successful. Thus, there is no direct evidence yet for the involvement of this protein in histidine transport. However, the available evidence strongly suggests that the histidine-binding protein is a component of the histidine permease system. The similarity in substrate specificities of the permease system and the binding protein, i.e. a high specificity for histidine and a much lower one for arginine, suggests that the recognition site of the permease resides in the binding protein. Moreover, the affinity constant of the histidine permease system, when measured in the presence of an inhibitor of protein synthesis, is of the same order of magnitude as the dissociation constant (K_D = 1.5 μM) of the histidine-binding protein complex.

Recent evidence by Ames and Lever (9) lends strong support to the view that the histidine-binding protein is a component of the histidine permease system. They reported the isolation of two histidine-binding proteins from S. typhimurium, a major one representing 95% of the total histidine-binding activity and a minor one representing about 5% of the total activity. They also have isolated S. typhimurium mutants which lack the major binding protein and are defective in histidine transport. Moreover, these investigators isolated another class of mutants which have 5 times the normal level of the histidine-binding protein and exhibits an increased rate of histidine transport. In our studies, we have detected only one histidine-binding protein in the shock fluid from osmotically shocked S. typhimurium LT-2 cells. We believe that it corresponds to the major binding protein isolated by Ames and Lever (9). Although they have not as yet reported on the extensive purification or characterization of the binding proteins which they have isolated, the major one does show the same heat stability as the one that we have isolated and purified.

REFERENCES

Purification and Characterization of a Histidine-binding Protein from Salmonella typhimurium LT-2 and Its Relationship to the Histidine Permease System
Barry P. Rosen and Frank D. Vasington


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