Identification of the Polyamine-induced Protein as a Periplasmic Oligopeptide Binding Protein*

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The physiological function of the polyamine-induced protein (PI protein), whose synthesis is stimulated at an early stage after the addition of putrescine to growing cells of a polyamine-requiring mutant of Escherichia coli (Mitsui, K., Igarashi, K., Kakegawa, T., and Hirose, S. (1984) Biochemistry 23, 2679–2683), has been studied. The following findings clearly show that the PI protein is a binding protein of an oligopeptide transport system. (a) PI protein was found in a periplasmic fraction. (b) When the restriction map of a clone for the PI protein gene was compared with Kohara's physical map (Kohara, Y., Akiyama, K., and Isono, K. (1987) Cell 50, 495–508), the gene was found at 27 min of the E. coli chromosome, where genes for an oligopeptide transport system were located. (c) The clone contained a 1,629-nucleotide open reading frame encoding a 543-amino acid protein whose calculated M₀ was 60,901, and the predicted amino acid sequence from this open reading frame was quite similar to that of an oligopeptide binding protein of Salmonella typhimurium. (d) When the transport activity of a tripeptide, Gly-Leu-Cys, was measured in a polyamine-requiring mutant of E. coli growing both in the presence and absence of putrescine, the activity was higher in the cells growing in its presence. (e) Polyamine stimulation of cell growth was greater when an oligopeptide rather than corresponding amino acids was added to the medium. These results suggest that the polyamine stimulation of PI protein synthesis at the early stage after the addition of putrescine contributes to the polyamine stimulation of cell growth through the supply of nutrients.

From recent reviews, it is clear that polyamines have important physiological roles and are essential for normal cell growth (1, 2). Some of their proliferative effects probably are caused by the stimulation of nucleic acid and protein synthesis. To clarify the physiological significance of polyamines in bacteria, we investigated their effect on various functions with a polyamine-requiring mutant, MA261, whose growth is limited in their absence. In this strain, the addition of putrescine to the medium caused immediate stimulation of the syntheses of two proteins; one was ribosomal protein Sl (3) and the other a protein of M₀ = 62,000 named polyamine-induced protein (PI protein) (4). Since polyamine stimulation of these two protein syntheses was followed by polyamine stimulation of cell growth, it seems that the physiological function(s) of these two proteins may be very important for cell growth. Although the function of ribosomal protein Sl has been well characterized (5, 6), the function of PI protein is as yet not clear.

In the present work, we have isolated a clone for the PI protein gene and characterized the gene for the PI protein. From these studies, various evidence was obtained to indicate that the PI protein is a periplasmic binding protein of an oligopeptide transport system. The results demonstrate that the oligopeptide transport system plays an important role in bacterial growth and that the synthesis of a binding protein for oligopeptides is strongly dependent on polyamines.

EXPERIMENTAL PROCEDURES

Bacteria Strains and Culture Conditions—E. coli MA261 (speB speC gly leu thr) was kindly provided by Dr. W. K. Maas, New York University School of Medicine. For the growth of E. coli MA261, medium A, containing 4 g of glucose, 7 g of K₂HPO₄, 3 g of KH₂PO₄, 500 mg of sodium citrate, 1 g of (NH₄)₂SO₄, 100 mg of MgSO₄/7H₂O, 2 mg of thiamin, 10 mg of biotin, and 100 mg each of leucine, threonine, methionine, serine, glycine, and ornithine per liter of water, was used. For polyamine starvation, cells growing exponentially in LB medium were inoculated to medium A and allowed to grow for 24 h at 37 °C (approximately five generations). The resulting polyamine-depleted bacteria (A₅₇₀ = 0.03) were then cultivated in either the presence (0.1 mg/ml) or absence of putrescine. At a designated time, the cells were harvested and washed with Buffer A at 15,000 × g for 15 min. The cells were then harvested and washed with Buffer A (10 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 60 mM KCl, and 6 mM 2-mercaptoethanol) and used for the experiments. E. coli DR112 (7), kindly supplied by Dr. D. R. Morris, University of Washington, was grown in LB medium.

Purification of the PI Protein and Preparation of Immunoglobulin for the Protein—The PI protein was purified from the 100,000 × g supernatant of E. coli MA261 as described previously (4). Immunoglobulin for the PI protein was prepared as described previously (8) and partially purified from the antiserum by precipitation with 40% saturated (NH₄)₂SO₄.

Measurement of PI Protein Synthesis by an Immunoprecipitation Method—E. coli MA261 was grown in the polyamine-deficient medium. When A₅₇₀ reached 0.1, the cells were divided into 5-ml aliquots and grown in the presence (0.1 mg/ml) or absence of putrescine. At a designated time, 0.05 ml of [³H]lysine (25 Ci/mmol) was added to each 5-ml aliquot, and the cells were allowed to grow for 20 min. They were then harvested and washed with Buffer A. The amount of PI protein synthesized was determined according to the method of Philipson et al. (9). To 400,000 cpm of [³H]lysine-labeled protein in 0.5 ml of Buffer B (10 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1% Triton X-100, and 0.1% SDS), 0.25 mg of immunoglobulin for

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The abbreviations used are: PI protein, polyamine-induced protein; SDS, sodium dodecyl sulfate; kdp, kilobase pair(s).
the PI protein was added, and the preparation was mixed gently for 60 min at room temperature. Then, 0.08 ml of 10% formalin-fixed Staphylococcus aureus Cowan I cells (Calbiochem) was added, and the preparation was mixed for 60 min at room temperature. After centrifugation, the precipitate was suspended in 0.04 ml of the gel sample buffer (62.5 mM Tris-HCl, pH 6.8, 5% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromphenol blue). The tube was boiled for 4 min, and the precipitate was discarded. Gel electrophoresis (10) and fluorography (11) were performed with a 0.03 ml aliquot. The relative intensity of the band on fluorography was traced with a Shimazu dual-wavelength TLC scanner CS910.

**Determination of Intracellular Localization of PI Protein—**Fractionation of cellular proteins into 100,000 × g supernatant, ribosomes, inner and outer membranes, periplasm, and residual was performed according to the method of Oliver and Beckwith (12). The amount of PI protein in each cellular fraction was measured by Western blotting (13). After electrophoretic transfer of the proteins, the nitrocellulose blot was saturated by incubation with 5% bovine serum albumin (Fraction V, Sigma) in TBS (150 mM Tris-HCl, pH 7.4) for 2 h at 37 °C. The blot was subsequently incubated overnight at 4 °C in TBS containing 10% newborn calf serum, 5% bovine serum albumin, and 0.06 mg/ml immunoglobulin for the PI protein. The blot was then rinsed several times with TBS containing 0.05% Nonidet P-40 and incubated with a 1:1,000 dilution of goat anti-rabbit antibody conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories) for 2 h at room temperature in TBS containing 10% newborn calf serum and 3% bovine serum albumin while being agitated. The blot was then rinsed several times with TBS containing 0.05% Nonidet P-40 and stained in 10 ml of TBS containing 0.018% (w/v) 4-chloro-1-naphthol (Merck) and 0.015% H₂O₂. The staining reaction was stopped by rinsing the blot with distilled water.

**Cloning and DNA Sequencing of the Gene for the PI Protein—**Total chromosomal DNA, prepared from E. coli DR112 according to the method of Rodriguez and Tait (14), was partially digested by Sau3AI. The 8-15-kb DNA fragments separated by agarose gel electrophoresis (22) were transferred to nitrocellulose paper by the method of Helfman et al. (17). The clone producing the PI protein was finally identified by SDS-polyacrylamide gel electrophoresis. The DNA was sequenced primarily by the dideoxy method of Sanger et al. (18), using the M13 phage system (23). The overproduction of the PI protein in cells containing pPI5 was shown to the samples from the supernatant, ribosomes, inner and outer membranes, periplasm, and plasma membrane. The amount of PI protein was determined by Western blot analysis. As shown in Fig. 2, the PI protein existed mainly in the periplasmic fraction. Only small amounts of the PI protein were observed in the ribosomal and supernatant fractions. The analysis of the proteins in the periplasmic fraction by staining with Coomassie Brilliant Blue R-250 showed that the PI protein was the most abundant protein in this fraction (data not shown).

**Cloning of the Gene for the PI Protein—**This was performed with the aid of anti-IGG for the PI protein as described under "Experimental Procedures," and two clones labeled pPI57 and pPI5 were obtained. They had inserts of 11.7 kbp and 7.8 kbp, respectively, and had an identical region of 6.8 kbp (Fig. 3). The overproduction of the PI protein in cells containing pPI5 was shown to be the samples from the supernatant, ribosomes, inner and outer membranes, periplasm, and plasma membrane. The amount of PI protein was determined by Western blot analysis. As shown in Fig. 2, the PI protein existed mainly in the periplasmic fraction. Only small amounts of the PI protein were observed in the ribosomal and supernatant fractions. The analysis of the proteins in the periplasmic fraction by staining with Coomassie Brilliant Blue R-250 showed that the PI protein was the most abundant protein in this fraction (data not shown).

**Figure 1.** Putrescine stimulation of cell growth and of PI protein synthesis in a polyamine-requiring mutant, MA261. E. coli MA261 was grown in the presence or absence of putrescine (0.1 mg/ml). Cell growth was followed by measuring AM. Measurement of PI protein synthesis was carried out at 20-min intervals up to 120 min as described under "Experimental Procedures." The ratio with or without putrescine was expressed as the "stimulation degree by putrescine."

**Figure 2.** Intracellular localization of PI protein. Fractionation of E. coli MA261 cells was performed as described under "Experimental Procedures." Odd and even numbers show the samples from the cells grown without putrescine and with putrescine, respectively. 1 and 2, periplasmic protein; 3 and 4, outer membrane protein; 5 and 6, inner membrane protein; 7 and 8, ribosomal protein; 9 and 10, cytoplasmic protein; 11 and 12, residual protein. To each lane, 1.5 µg of protein was applied.
at 27 min. A gene for an oligopeptide binding protein (Mr = 60,000), which is located in the periplasmic space, has been mapped at 27 min (24, 25). Thus, the PI protein is probably an oligopeptide binding protein encoded by OppA (26).

**Nucleotide Sequence of the Gene Encoding the PI Protein—**

To identify the PI protein as an oligopeptide binding protein, we tried to determine the nucleotide sequence of the gene for the protein. A subclone, pPI5.1, which has the insert of 3.8 kbp, was obtained by deleting the SalI fragment of pPI5 (Fig. 3). The pPI5.1 overproduced the PI protein greatly (Fig. 4). Using this plasmid, pPI5.1, the nucleotide sequence of the gene encoding the PI protein was determined. Fig. 5 shows the nucleotide sequence and the deduced amino acid sequence of the PI protein. It contained the candidates for -10 and -35 regions of the promoter and Shine-Dalgarno sequence (28), and transcriptional termination signal (29). The open reading frame consisted of 543 amino acids, and its molecular weight was estimated as 60,901 (Fig. 6).

**Characteristics of the Purified PI Protein—**

To determine the processing site of the PI protein by a signal peptidase, the N-terminal amino acid sequence was determined by Edman degradation. As shown in Fig. 7, the N-terminal 20-amino acid sequence was exactly the same as the deduced 27th to 46th amino acid sequence from the nucleotide sequence of the PI protein gene. These results show that the processing site is between Ala-26 and Ala-27 (Figs. 5 and 6). Amino acid compositions obtained from amino acid analysis of the purified PI protein and from deduced amino acids from the gene for the PI protein were compared. As shown in Table I, the compositions were quite similar, indicating that the open reading frame of the PI protein gene really encodes the PI protein.

The nucleotide sequence and the deduced amino acid sequence of an oligopeptide binding protein of *Salmonella typhimurium* have already been reported (26). The homology of the two nucleotide sequences and the deduced amino acid sequences was 75% and 84%, respectively. These results indicate that the *E. coli* PI protein is an oligopeptide binding protein.

Some Characteristics of *E. coli* MA261 Grown in the Presence of a Tripeptide, Gly-Leu-Tyr—

It has been reported that Gram-negative bacteria such as *E. coli* and *S. typhimurium* are able to utilize a wide variety of small peptides as nutrients (30). The Gly-Leu-Tyr uptake activity was measured with *E. coli* MA261 grown in the presence of Gly-Leu-Tyr with or without putrescine (0.1 mg/ml). As shown in Fig. 8, the Gly-Leu-Tyr uptake activity was higher in *E. coli* MA261 grown with putrescine than in that without. When *E. coli* Q13, which is normal in polyamine biosynthesis, was used instead of *E. coli* MA261.
Function of Polyamine-induced Protein

Fig. 6. Comparison of amino acid sequences of PI protein in E. coli (A) and the oligopeptide binding protein in S. typhimurium (B). The amino acid sequence of the oligopeptide binding protein in S. typhimurium was taken from Ref. 26. The arrow (↓) indicates the cutting site by a signal peptidase, and stars (☆) indicate the homologous amino acid in the two proteins.

Fig. 7. Comparison of N-terminal amino acid sequences deduced from the nucleotide sequence of the gene for PI protein and determined by Edman degradation analysis. The upper, middle, and lower lines show the nucleotide sequence, deduced amino acid sequence, and determined amino acid sequence by Edman degradation, respectively. The 21st and 22nd amino acids of PI protein could not be identified in Edman sequencing.

TABLE I
Amino acid composition of PI protein

<table>
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<tr>
<th>Amino acid</th>
<th>Amino acid analysis</th>
<th>Nucleotide sequence</th>
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<tr>
<td>Alamine</td>
<td>9.09</td>
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<tr>
<td>Asparagine</td>
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<tr>
<td>Aspartic acid</td>
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<td>Arginine</td>
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<td>0.39</td>
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<tr>
<td>Cysteine</td>
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<tr>
<td>Glutamine</td>
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</tr>
<tr>
<td>Glutamic acid</td>
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<td>2.32</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.26</td>
<td>2.26</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.60</td>
<td>6.18</td>
</tr>
<tr>
<td>Leucine</td>
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<td>7.72</td>
</tr>
<tr>
<td>Lysine</td>
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<td>1.54</td>
</tr>
<tr>
<td>Methionine</td>
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<tr>
<td>Phenylalanine</td>
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<tr>
<td>Proline</td>
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<td>6.60</td>
</tr>
<tr>
<td>Threonine</td>
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<td>7.53</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>4.40</td>
<td>5.60</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.47</td>
<td>7.72</td>
</tr>
</tbody>
</table>

Fig. 8. The tripeptide, Gly-Leu-Tyr, uptake activity of E. coli MA261 grown in the presence and absence of putrescine (0.1 mg/ml). The uptake activity was measured as described under "Experimental Procedures." 0, the uptake activity of E. coli MA261 grown in the presence of putrescine; O, the uptake activity of E. coli MA261 grown in the absence of putrescine.

TABLE II
Effect of an oligopeptide or corresponding amino acids on the cell growth of E. coli MA261

<table>
<thead>
<tr>
<th>Amino acids or oligopeptide in medium</th>
<th>Doubling time (min)</th>
<th>Ratio (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Putrescine</td>
<td>195±15</td>
<td>1.46</td>
</tr>
<tr>
<td>-Putrescine</td>
<td>285±32</td>
<td>1.94</td>
</tr>
</tbody>
</table>

coli MA261, putrescine did not stimulate the Gly-Leu-Tyr uptake activity (data not shown).

The growth of E. coli MA261 was faster in the presence of the tripeptide, Gly-Leu-Tyr, than in the presence of the corresponding amino acids. In addition, polyamine stimulation of cell growth was greater in the presence of the tripeptide than in the presence of the corresponding amino acids (Table II). These results indicate that E. coli prefers oligopeptides to amino acids, meaning that this bacterium has a high level oligopeptide transport system, a result of the intracellular activity of polyamines.

DISCUSSION

It is well known that the synthesis of some specific proteins is stimulated by polyamines at the level of initiation of translation in cell-free systems (31-34). This has also been demonstrated in vivo protein synthesis. In a polyamine-requiring mutant of E. coli, polyamine stimulation of the syntheses of both ribosomal protein S1 (3) and PI protein (4) was followed by the stimulation of cell growth. Since the S1 protein can stimulate MS2 RNA- and poly(U)-dependent polypeptide syntheses (5, 6, 35), the increase of S1 protein content in cells appears to be one of the essential events for the stimulation of cell growth. The activation of ribosomes was achieved by the association of the S1 protein to S1-depleted ribosomes at a lower than 1:1 molar ratio.

In this study, we have investigated the physiological function of the PI protein. Experimental data clearly show that the PI protein is an oligopeptide binding protein (a protein encoded by OppA (26)). E. coli MA261 grew faster in the presence of an oligopeptide than the corresponding amino
acids. The increment in cell growth induced by the oligopeptide was much more significant in the presence of polyamines in the polyamine-requiring mutant, since the level of the oligopeptide transport system was elevated drastically by the addition of polyamines. These results suggest that the polyamine stimulation of PI protein synthesis at the early stage after the addition of putrescine contributes to the polyamine stimulation of cell growth through the supply of nutrients. It has been reported that the oligopeptide transport system not only plays an important nutritional role but also is required for the recycling of cell wall peptides (36). The oligopeptide transport mutants which were unable to recycle cell wall peptides showed growth defects under certain conditions (36). Therefore, polyamines may also contribute to cell wall synthesis. In the previous communication (4), we demonstrated that the PI protein could stimulate the synthesis of MS2 RNA replicase in the presence of polyamines. However, that result was an artifact, as the PI protein is known not to exist in the cytoplasm.

We are now studying the biochemical mechanism by which the PI protein synthesis is stimulated by putrescine. We presume that polyamines can recognize specific nucleotide sequences of the PI protein gene and/or its mRNA. The polyamine-induced change of the tertiary structure of the nucleotides is probably important for the putrescine stimulation of PI protein synthesis. Our preliminary experiments suggest that polyamine regulation may take place mainly at the translational level.

The number of amino acids of the processed PI protein (an oligopeptide binding protein) was exactly the same (517 residues) in E. coli and S. typhimurium (Fig. 6). However, a difference in the number of amino acid residues in the signal peptides was observed: 26 amino acids in E. coli and 25 amino acids in S. typhimurium. In addition, the homology of amino acids in the two species was lower in the portion of the signal peptides than in that of the oligopeptide binding protein. The functioning portion of the protein can be supposed to be well conserved.

When pPI5 was subcloned to pPI5.1, the overproduction of the PI protein was greatly enhanced (Fig. 4). In case of pPI5.1, some portion of the genes for OppB, OppC, OppD, and OppF was probably deleted. The overproduction of the membrane-associated BCDF proteins is probably deleterious to cells. It would be of interest to find out how the expression of the whole gene of the oligopeptide transport system is regulated.

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Identification of the polyamine-induced protein as a periplasmic oligopeptide binding protein.
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