Polyamine Stimulation of the Synthesis of Oligopeptide-binding Protein (OppA)

IN Volvement of a Structural Change of the Shine-Dalgarno Sequence and the Initiation Codon AUG in OppA mRNA*

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We previously suggested that the degree of polyamine stimulation of oligopeptide-binding protein (OppA) synthesis is dependent on the secondary structure and position of the Shine-Dalgarno (SD) sequence on mRNA. To study the structural change of OppA mRNA induced by polyamines and polyamine stimulation of initiation complex formation, four different 130-mer OppA mRNAs containing the initiation region were synthesized in vitro. The structural change of these mRNAs induced by polyamines was examined by measuring their sensitivity to RNase T1, specific for single-stranded RNA, and RNase V1, which recognizes double-stranded or stacked RNA. In parallel, the effect of spermidine on mRNA-dependent fMet-tRNA binding to ribosomes was examined. Our results indicate that the secondary structure of the SD sequence and initiation codon AUG is important for the efficiency of initiation complex formation and that spermidine relaxes the structure of the SD sequence and the initiation codon AUG. The existence of a GC-rich double-stranded region close to the SD sequence is important for spermidine stimulation of fMet-tRNA binding to ribosomes. Spermidine apparently binds to this GC-rich stem and causes a structural change of the SD sequence and the initiation codon, facilitating an interaction with 30 S ribosomal subunits.

Polyamines (putrescine, spermidine, and spermine) are necessary for normal cell growth, and their proliferative effects are probably due to stimulation of nucleic acid and protein synthesis (1, 2). We previously reported that polyamines can stimulate the synthesis of some types of prokaryotic and eukaryotic proteins in cell-free systems (3, 4) and in vivo (5, 6) and that the assembly of 30 S ribosomal subunits is stimulated by polyamines (7, 8). We also found that most polyamines exist as a polyamine-RNA complex in cells (9). In a rabbit reticulocyte cell-free system, under conditions in which spermidine pro-

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1 The abbreviations used are: PI, polyamine-induced; OppA, oligopeptide-binding protein; SD, Shine-Dalgarno; PCR for polymerase chain reaction.
and III were pMW211, pMW9-lacZ, and pMWSD2, respectively, in addition to BlueScript II SK+. Primers used for the first PCR were P2/P3 and P4/P1 for construct I, P2/P3 and P4/P9 for construct II, and P2/P5 and P1/P6 for construct III. Then, second PCRs for constructs I and III and for construct II were performed using the first PCR products as templates and P1/P2 and P2/P9 as primers, respectively. These PCR products thus obtained with Nhel and HindIII and inserted into the same restriction sites of pSP64 (Promega). PI-SD* (IV) was similarly made using construct I as the template. The first PCR was performed using P2/P8 and P1/P7 as primers, and the second PCR was performed using the first PCR product as the template and P1/P2 as the primer. The product was inserted into pSP64 as described above. The nucleotide sequence of the four DNAs was confirmed by the DNA sequencer DSQ-1000 (Shimadzu, Japan). Four kinds of 130-mer OppA mRNAs (PI-130, PI-lacZ, PI-SD2, and PI-SD*) were synthesized from T7 promoter-fused constructs I, II, III, and IV, respectively, by T7 RNA polymerase (TOYOBO, Japan) according to the manufacturer’s instructions. The DNAs were linearized by HindIII prior to the reaction.

PI-71 mRNA was prepared by T7 RNA polymerase using a single-stranded PI-71 DNA template and T7 promoter primer (5'-TATAGC-ACCTCCTATA-3').

**Assay for fMet-tRNA Binding to Ribosomes**—The reaction mixture (0.1 ml) containing 50 mM Tris-HCl, pH 7.2, 100 mM KCl, 1 mM dithiothreitol, 1 mM GTP, 90 μg (33.3 pmol) of salt-washed ribosomes, 14 μg of crude initiation factors, 18.7 pmol of [3H]fMet-tRNA, AUG or various kinds of OppA mRNA at the specified concentration, and Mg2⁺ and spermidine at the indicated concentrations was incubated at 30 °C for 4 min. The reaction mixture was passed through a cellulose nitrate filter (pore size, 0.45 μm) (ADVANTEC, Japan). The filter was washed with 10 ml of a buffer containing 50 mM Tris-HCl, pH 7.2, 100 mM KCl, and 10 mM Mg2⁺. Radioactivity on the filter was measured with a liquid scintillation spectrometer. The reaction was linear during incubation.

**Limited RNase Digestion of the 5'-32P-Labeled OppA mRNAs**—The reaction mixture (20 μl) for limited RNase digestion contained 50 mM Tris-HCl, pH 7.5, 2 mM Mg2⁺, 100 mM KCl, 5 × 10⁻⁶ cpm 5'-32P-labeled OppA mRNA, 2 μg of yeast tRNA (Sigma), and spermidine at the specified concentration and was incubated at 37 °C for 15 min. Then, 0.12 unit of RNase T₁ (Amersham Pharmacia Biotech) or 0.085 unit of RNase V₁ (cobra venom, Amersham Pharmacia Biotech) was added and incubated further at 30 °C for 15 or 10 min, respectively. The 5'-32P-labeled OppA mRNA used was prepared with [γ-32P]ATP (specific activity, 148 TBq/mmol) and T4 polynucleotide kinase (TOYOBO, Japan) and purified according to the method of Donis-Keller et al. (17). The reaction was stopped by the addition of 0.3 ml (30 μg) of yeast tRNA and 0.3 ml of phenol/chloroform/isoamyl alcohol (50:50:1). The RNA fragments were recovered from the water layer by ethanol precipitation and dissolved in 10 μl of a solution of 7 mM urea, 0.05% xylene cyanol, and 0.05% bromphenol blue. Gel electrophoresis was performed by the procedure of Donis-Keller et al. (17). Radioactivity was quantified using a Fuji BAS 2000 II imaging analyzer.

**Prediction of the Secondary Structure of RNA**—Optimal computer folding of mRNAs was performed by the method of Zucker and Stiegler (18). Free energy (∆G) for the formation of the secondary structure was calculated on the basis of the data of Turner and Sugimoto (19).

**RESULTS**

**Determination of the Size of OppA mRNA Is Necessary for the Efficient Recognition of the SD Sequence**—We have shown that the SD sequence of OppA mRNA is absolutely necessary for OppA synthesis (14). Gualerzi and co-workers (20) showed that the SD sequence is necessary for efficient initiation complex formation using an artificial 126-mer mRNA. However, the size of mRNA necessary for efficient recognition of the SD sequence has not been determined. Therefore, we initially carried out experiments to determine the size of OppA mRNA necessary for efficient recognition of the SD sequence.

Several different OppA mRNAs of different sizes and with differences in the position of the SD sequence were synthesized (Fig. 1). The effects of these mRNAs on fMet-tRNA binding to ribosomes were measured by adding increasing amounts of mRNA. In Table I, the relative activities of these mRNAs are shown as a function of the amount of mRNA that caused maximal binding of fMet-tRNA to ribosomes. In the case of AUG, 90 times more mRNA (3 nmol) than ribosomes (33 pmol) and 11 mM Mg2⁺ were necessary for maximal binding. When the SD sequence was fused to the initiation codon AUG (PI-25), maximal fMet-tRNA binding was obtained in a molar ratio for mRNA and ribosomes of 30 to 1 (i.e., the affinity of mRNA for ribosomes increased by 3-fold), and the optimal Mg2⁺ concentration decreased to 9 mM. When the SD sequence was positioned only five nucleotides upstream from the initiation codon AUG (PI-19), the maximal binding activity was almost the same as that seen with PI-25, but the affinity for ribosomes decreased compared with PI-25. The results suggest that a high concentration of Mg2⁺ (9–11 mM) is necessary for small mRNAs to direct fMet-tRNA binding to ribosomes. With PI-71, the affinity for ribosomes increased greatly, and the optimal Mg2⁺ concentration decreased to 6 mM. However, the maximal

**Table I**

| mRNA | mRNA/Ribosomes | Optimal Mg²⁺ | fMet-tRNA bound |
|------|----------------|-------------|-----------------|
| AUG  | 90             | 11          | 2.12            |
| PI-19| 100            | 9           | 1.27            |
| PI-25| 30             | 9           | 1.31            |
| PI-71| 6              | 5           | 2.91            |
| PI-130| 3              | 4           | 9.14            |
fMet-tRNA binding to ribosomes was still 2.91 pmol. With PI-130 mRNA, an increase in affinity for ribosomes, a lowering of the optimal Mg\(^{2+}\) concentration, and a large increase in maximal fMet-tRNA binding were observed. The results suggest that more than 100 nucleotides are required for efficient recognition of the OppA SD sequence during initiation complex formation.

Effects of Spermidine on fMet-tRNA Binding to Ribosomes Directed by AUG and Various OppA mRNAs—The effects of spermidine on fMet-tRNA binding to ribosomes were examined in the presence of different concentrations of Mg\(^{2+}\). Binding of fMet-tRNA to ribosomes directed by AUG, PI-19, and PI-25 was slightly stimulated by 1–2 mM spermidine (approximately 1.1-fold) in the presence of 6–11 mM Mg\(^{2+}\), and higher concentrations of spermidine did not influence fMet-tRNA binding (data not shown). When PI-71 was used as the mRNA, spermidine significantly stimulated fMet-tRNA binding to ribosomes, but 4–6 mM spermidine was necessary to cause stimulation (Fig. 2). It is noteworthy that PI-71 includes a GC-rich stem I structure (see Fig. 3), which was suggested to be the binding site of spermidine in a previous publication (14).

When PI-130 was used as the mRNA instead of PI-71, 0.5–1 mM spermidine caused a stimulation of fMet-tRNA binding to ribosomes in the presence of 1–3 mM Mg\(^{2+}\) (Fig. 2). A decrease in the optimal Mg\(^{2+}\) and spermidine concentrations was observed with the increasing size of the OppA mRNA, suggesting that low concentrations of Mg\(^{2+}\) and spermidine may be enough to maintain a suitable conformation of long mRNAs. We examined the effect of spermidine on fMet-tRNA binding to ribosomes directed by other 130-mer OppA mRNAs. These
experiments were designed to clarify the structural change of the OppA mRNA induced by polyamines that is necessary for stimulation of Met-tRNA binding to ribosomes. PI-lacZ mRNA consists of the 5'-untranslated region of OppA mRNA fused with the open reading frame of lacZ, so that the GC-rich stem I structure is modified. In PI-SD2, the triple Gs in the GC-rich stem I were replaced by triple Us, and in PI-SD* the SD sequence was positioned at the typical site, seven nucleotides upstream from the initiation codon AUG (Fig. 1). When PI-lacZ, PI-SD2, and PI-SD* were used as mRNAs, 0.5–1 mM spermidine...
dine slightly stimulated fMet-tRNA binding in the presence of 2–3 mM Mg²⁺ (Fig. 2). The maximal fMet-tRNA binding to ribosomes in the presence of spermidine was PI-130 > PI-SD* > PI-lacZ > PI-SD2. Thus, the levels of both polyamine stimulation and of initiation complex formation were parallel with the effects of these mRNAs on protein synthesis previously determined in vivo (14).

It has been reported that the equilibrium between 70 S ribosomes and the 30 S and 50 S subunits is influenced by polyamines (21, 22). In the presence of 2–3 mM Mg²⁺ and 100 mM K⁺, however, the percentage of 70 S ribosomes (70–80% of total ribosomes) was not influenced strongly by 0.5–1 mM spermidine (data not shown).

Changes of Secondary Structure of the SD Sequence and the Initiation Codon AUG of OppA mRNA by Spermidine—The structural change of OppA mRNA induced by spermidine was examined by limited digestion of the mRNA with a single-stranded G-specific RNase T₁. The digested products were analyzed by gel electrophoresis. When PI-71 mRNA was used as a substrate, the G in the initiation codon AUG was easily hydrolyzed in the absence or presence of spermidine (Fig. 3). The five Gs in the SD sequence were hydrolyzed at higher concentrations of spermidine. The optimal concentration of spermidine for the hydrolysis of the SD sequence was 5–10 mM, in accordance with the results of fMet-tRNA binding to ribosomes (see Fig. 2). The results indicate that a structural change of the SD sequence of PI-71 mRNA by polyamines is important for efficient fMet-tRNA binding to ribosomes.

The RNase T₁ sensitivity of the different 130-mer OppA mRNAs was studied to determine the relationship between the structural change of the OppA mRNAs and polyamine stimulation of fMet-tRNA binding to ribosomes. When PI-130 mRNA was used as a substrate, hydrolysis of the five Gs in the SD sequence and the G in the initiation codon AUG was stimulated greatly by spermidine (Fig. 4). The optimal concentration of spermidine was 0.4–0.8 mM. The hydrolysis of Gs positioned at 18, 20, and 23 (see Fig. 6) was not influenced by spermidine. The results suggest that spermidine relaxes the structure of both the SD sequence and the initiation codon AUG of PI-130 mRNA.

When PI-lacZ mRNA was used as a substrate, the hydrolysis of five Gs in the SD sequence was inhibited by spermidine, although the hydrolysis of the G in the initiation codon AUG was stimulated by spermidine. When PI-SD2 mRNA was used as a substrate, the five Gs in the SD sequence were relatively insensitive to RNase T₁, and hydrolysis of the G in the initiation codon AUG was not influenced by spermidine. When PI-SD* mRNA was used as a substrate, the five Gs in the SD sequence and the G in the initiation codon AUG were strongly hydrolyzed, and spermidine did not influence this hydrolysis (Fig. 4). The putative secondary structures of the initiation regions of the four mRNAs are shown in Fig. 4.

Changes of Secondary Structure of the Region between the SD Sequence and the Initiation Codon AUG of OppA mRNA by Spermidine—OppA mRNA has an unusual property in that the SD sequence is 12 nucleotides upstream from the initiation codon AUG. Thus, the SD sequence and the initiation codon become closer during formation of the initiation complex and that this is important for initiation. There are no G residues in the region between the SD sequence and the initiation codon.

![Fig. 6. Possible secondary structure of PI-130 and PI-lacZ mRNAs and their hydrolyzed points by RNase T₁ and RNase V₁. Initiation codon AUG is circled, and the SD sequence is boxed. Continuous GC stems (stem I) and their modified stems (stem I*) are shaded. SPD, spermidine.](image-url)
Therefore, to look for structural changes of this region induced by spermidine, we studied sensitivity to RNase V₁, which recognizes double-stranded RNA or stacked RNA (23). As shown in Fig. 5, the region between the SD sequence and the initiation codon AUG of PI-130 and PI-lacZ became sensitive to RNase V₁ in the presence of spermidine, whereas the sensitivity to RNase V₁ of this region in PI-SD2 and PI-SD⁺ was weak and did not change in the presence of spermidine. The results suggest that the relative distance between the SD sequence and the initiation codon AUG becomes shorter in PI-130 and PI-lacZ but not in PI-SD2 and PI-SD⁺, because the loop I structure of the former two mRNAs adopts a stacking conformation. The SD sequence of PI-SD2 was hydrolyzed by RNase V₁ (Fig. 5), indicating that this region makes a stem structure (see Fig. 4).

The Relationship between Spermidine Stimulation of Initiation Complex Formation and Structural Change of OppA mRNAs by Spermidine—The structural change of OppA mRNAs is summarized in Fig. 6 and Table II. Among four OppA mRNAs, only fMet-tRNA binding to ribosomes directed by PI-130 mRNA was stimulated by spermidine (Fig. 2). In that case, the SD sequence and the initiation codon AUG in the mRNA were relaxed by spermidine, and the relative distance between the SD sequence and the initiation codon AUG became shorter, judging from the increased sensitivity of the loop I structure to RNase V₁.

With PI-lacZ, the stem structure of the SD sequence was strengthened by spermidine, but the structure of the initiation codon AUG became loosened by spermidine. Furthermore, the relative distance between the SD sequence and the initiation codon AUG became shorter. Thus, the two opposing effects of spermidine largely cancel each other, and spermidine has only a small net effect on PI-lacZ. With PI-SD2 and PI-SD⁺, spermidine does not produce a structural change of the mRNAs, and fMet-tRNA binding to ribosomes was therefore not influenced by spermidine. Because the SD sequence of PI-SD⁺ is exposed in the absence of spermidine, relatively effective fMet-tRNA binding to ribosomes can occur in the absence of spermidine.

DISCUSSION

It is known that the SD sequence, the initiation codon AUG, and the spacing between these two elements are important determinants of the efficiency of translational initiation (24). In a previous publication (14) we studied the molecular mechanism of polyamine stimulation of OppA synthesis in a polyamine-requiring mutant of E. coli using several artificial oppA genes. Through analysis of the secondary structure by optimal computer folding of OppA mRNA, it was suggested that the degree of polyamine stimulation of OppA synthesis is dependent on the secondary structure of the SD sequence in addition to its position.

In this communication, we synthesized four different 130-mer OppA mRNAs and studied the relationship between spermidine stimulation of fMet-tRNA binding to ribosomes and structural changes of OppA mRNA. We found that the strong polyamine stimulation of protein synthesis directed by natural OppA mRNA is caused by exposure of the SD sequence and the initiation codon AUG due to polyamine binding to the GC-rich stem close to the SD sequence. On the other hand, the lack of effect of polyamines on protein synthesis directed by an artificial OppA mRNA (9-lacZ mRNA, including PI-lacZ initiation region) was due to stronger base pair formation of the SD sequence, perhaps because of direct polyamine binding to the stem/bulge structure containing the SD sequence (Fig. 6).

It is known that spermidine can bind to RNA at a ratio of two to three molecules per 100 nucleotides of RNA (6, 9, 25). It is expected that spermidine binds to the four 130-mer mRNAs that we have studied at about the same ratio. However, the structural change induced by spermidine is different for each mRNA. Spermidine may recognize a special structure or sequence to cause a drastic structural change in the native OppA mRNA. One candidate is a bulge structure containing a GC-rich stem, like stem I in PI-130 mRNA. Such a stem structure was strengthened by spermidine (Fig. 6). In this respect, we have previously reported that only Ile-tRNA formation was stimulated by spermidine among 20 aminoacyl-tRNA formations (26, 27). The amino acid acceptor stem of tRNAIle, which is responsible for polyamine stimulation, also consists of a GC-rich stem containing one unpaired G similar to the bulge structure, and the amino acid acceptor stem structure was strengthened by polyamines.

It is notable that more than 100 nucleotides are required for the SD sequence of OppA mRNA to be recognized efficiently during formation of the initiation complex. The results of this study support the idea that the region of mRNA required for the optimal initiation complex formation is considerably larger than that protected by ribosomes.

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