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Note

Requirement for C-terminal Extension to the RNA Binding Domain for Efficient RNA Binding by Ribosomal Protein L2

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Ribosomal protein L2 is a primary 23S rRNA binding protein in the large ribosomal subunit. We examined the contribution of the N- and C-terminal regions of *Bacillus stearothermophilus* L2 (*Bst*L2) to the 23S rRNA binding activity. The mutant desN, in which the N-terminal 59 residues of *Bst*L2 were deleted, bound to the 23S rRNA fragment to the same extent as wild type *Bst*L2, but the mutation desC, in which the C-terminal 74 amino acid residues were deleted, abolished the binding activity. These observations indicated that the C-terminal region is involved in 23S rRNA binding. Subsequent deletion analysis of the C-terminal region found that the C-terminal 70 amino acids are required for efficient 23S rRNA binding by *Bst*L2. Furthermore, the surface plasmon resonance analysis indicated that successive truncations of the C-terminal residues increased the dissociation rate constants, while they had little influence on association rate constants. The result indicated that reduced affinities of the C-terminal deletion mutants were due only to higher dissociation rate constants, suggesting that the C-terminal region primarily functions by stabilizing the protein L2-23S rRNA complex.

Key words: *Bacillus stearothermophilus*; protein-RNA interaction; surface plasmon resonance; ribosomal protein L2

It is now established that the ribosome is a ribozyme, being 23S rRNA as a catalytic component,[1,2] although its catalytic mechanism is still controversial.[3–5] It is however also true that 23S rRNA alone is unable to catalyze a peptide bond formation but performs the catalytic function in close association with a few ribosomal proteins.[6] Hence, the 23S rRNA-ribosomal protein interaction is central to understanding the molecular mechanism of protein biosynthesis processes.

Ribosomal protein L2 is a primary 23S rRNA binding protein and known to be the most important constituent of the peptidyltransferase center.[7] In the foregoing study, the 23S rRNA binding domain was localized in the center of *Bacillus stearothermophilus* L2 (*Bst*L2) between amino acids 60 and 201.[8] This assignment was carried out by proteinase protection analysis of the *Bst*L2-23S rRNA complex; tryptic and chymotryptic digestions of the complex produced two protected fragments—positions 60–206 and 58–201. By virtue of this property, the central region from Gln60 to Leu201 in *Bst*L2 was referred to as the *Bst*L2-23S rRNA binding domain (*Bst*L2-RBD). It was however found by a filter binding assay that the *Bst*L2-RBD was unable to bind to the 23S rRNA fragment (positions 1791–1865) containing the *Bst*L2 binding site.[9] This result indicated that the N- and/or C-terminal extension(s) to the *Bst*L2-RBD may be required for efficient RNA binding by *Bst*L2. To gain more insight into the understanding of the interaction mechanism of protein L2 and 23S rRNA, we have attempted to define the 23S rRNA binding region in *Bst*L2.

Our strategy was first to produce two *Bst*L2 mutant proteins (desN and desC), in which N-terminal (pos. 1～59) and C-terminal (202～275) regions were deleted, respectively, and then they were characterized with respect to their 23S rRNA binding activity using the filter binding assay. Next, based on the results obtained by analysis of desN and desC, the putative RNA binding region was further gradually deleted to define a minimum RNA binding region. Finally, the contribution of the terminal extension to *Bst*L2-RBD was quantitatively examined by surface plasmon resonance (SPR) using the BIAcore™ system.

The corresponding genes encoding the deletion mutants desN and desC were amplified by PCR using specific primers from the cloned *Bst*L2 gene.[10] For overproduction of *Bst*L2 and its mutant proteins, the

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Abbreviations: *Bst*L2, ribosomal protein L2 from *Bacillus stearothermophilus*; RBD, 23S rRNA binding domain; *Hma*L2, ribosomal protein L2 from *Haloarcula marismortui*; SPR, surface plasmon resonance

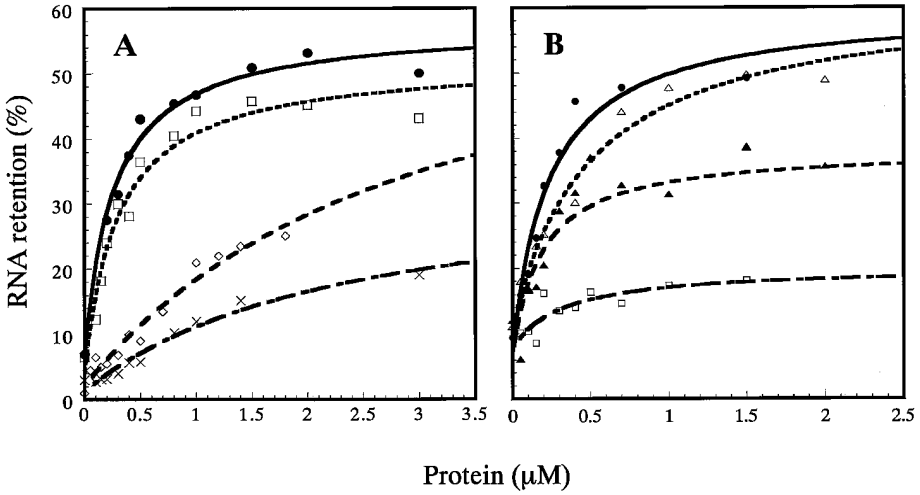

Fig. 1. Saturation Binding Curves for the Interaction of *Bst*L2 and Its Mutant Proteins with the 23S rRNA Fragment.

Increasing amounts of proteins were incubated with the 23S rRNA fragment and isolated by filtration. A, binding curves for *Bst*L2 (●), desN (□), desC (◇), and *Bst*L2-RBD (×); B, binding curves for *Bst*L2 (●), dN270 (△), dN260 (▲), and dN250 (□).

genes were placed under the control of the T7 phage promoter on the expression plasmid pET-22b,[11] induced with IPTG, and the recombinant proteins thus produced were purified using an S-Sepharose column, as previously described.[10] The capability to bind to 23S rRNA was examined by the filter binding assay[12] using the *B. stearothermophilus* 23S rRNA fragment (positions 1791～1865) which was synthesized by *in vitro* transcription, as previously described.[10] A real-time analysis of interaction of *Bst*L2 or its mutants and 23S rRNA fragment was done by SPR with the BIAcore™ instrument, as described for the study on the interaction of ribosomal protein L5 and 5S rRNA.[13]

Figure 1 shows typical binding curves of the mutants. The mutant desN retained 23S rRNA binding activity to the same extent as wild type *Bst*L2, while the mutant desC severely abolished the binding activity (Fig. 1A). This indicated that the C-terminal extension to *Bst*L2-RBD is involved in the 23S rRNA binding activity of *Bst*L2. We next prepared three mutant proteins, dN270, dN260, and dN250, in which 5, 15, and 25 amino acid residues from the C-terminus were deleted, respectively, and the resulting mutant proteins were again analyzed by a filter binding assay. The result showed that deletion of 5 residues had no significant effect on the binding activity, while those of 15 and 25 amino acids gradually weakened the binding activity to 23S rRNA (Fig. 1B). The result indicated that the C-terminal 70 amino acids extension to *Bst*L2-RBD is required for efficient 23S rRNA binding by *Bst*L2.

Next, the contribution of the C-terminal region to the 23S rRNA binding activity was quantitatively analyzed by SPR with the BIAcore™ system. Figure 2 shows typical sensorgrams of the association and dissociation of *Bst*L2 and its mutant proteins. The values for the association rate constant (k_{on}), dissociation rate constant (k_{off}), and association constant (K_a) obtained are summarized in Table 1. These measurements showed that three mutants protein retained values for the association rate constants (k_{on}) comparable to that of wild type, while the mutants dN260 and dN250 showed increased values for the dissociation rate constants (k_{off}). This indi-

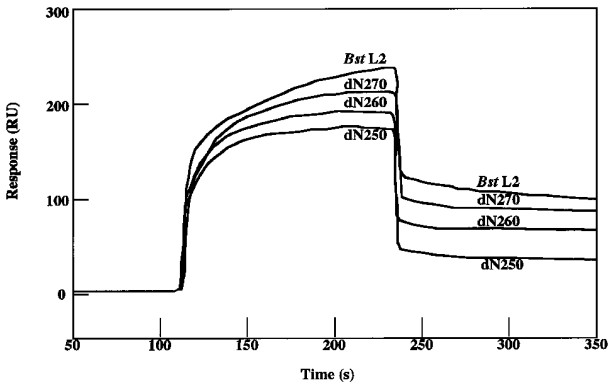

Fig. 2. Sensorgrams of Real Time Analyses by BIAcore of the Interaction of *Bst*L2 and Its Mutant Proteins with 23S rRNA Fragment.

The SA sensor chips (BIAcore Inc.) were coated with a low concentration (about 60 resonance units) of a biotinylated 19-nucleotide oligonucleotide (5′-CTCCCATATGGCAGGTGTC-3′) complementary to the nucleotide sequence at the 3′ end of the *B. stearothermophilus* 23S rRNA fragment.[10] Five hundred nM solution of the 23S rRNA fragment in 1 M NaCl and TMK buffer (50 mM Tris-acetate buffer, pH 7.6, containing 20 mM Mg-acetate, 350 mM KCl, and 6 mM β-mercaptoethanol) was captured on a flow cell by manually injecting at a 2 μl/min flow rate. Small amounts of 23S rRNA fragment were used to coat the surface (about 80 resonance units). No 23S rRNA fragment was captured on flow in another cell, so it could be used as a reference surface. The biosensor assay was run at 37°C in TMK buffer and 10% glycerol. The proteins were injected over flow cells for 2 min at concentration of 2 μM using a flow rate of 30 μl/min. The bound protein was removed with a 60 s wash with 2 M NaCl.

Table 1. Kinetic Contants for the Interaction of *Bst*L2 or Its Mutants with RNA

Protein	Surface plasmon resonance measurement		
	K_a ($\times 10^6$ M^{-1})	k_{on} ($\times 10^5$ M^{-1} S^{-1})	k_{off} ($\times 10^{-3}$ S^{-1})
*Bst*L2	4.19	5.33	1.27
dN250	0.28	4.08	14.30
dN260	0.55	4.03	7.24
dN270	1.88	4.01	2.13

cates that the lower binding activity of the C-terminal deletion mutants dN250 and dN260 was due almost exclusively to the increased dissociation rate constants. It is thus suggested that the C-terminal region contributes to 23S rRNA binding primarily by keeping *Bst*L2 in direct contact with 23S rRNA once the complex has been formed.

The crystal structure of the *Haloarcula marismortui* 50S subunit showed the terminal structure of the protein L2 (*Hma*L2) to be highly extended from the center of the molecule.[1] Examining the structure of *Hma*L2 in the 50S subunit, the C-terminal extension seems to be far away from the center of the molecule. As previously described, the C-terminal region (pos. 201 ~ 275) of *Bst*L2 is highly accessible to proteolysis in the complex with 23S rRNA.[8] Taken this information into consideration, it is unlikely that the C-terminal region makes direct contact with 23S rRNA. Rather, it can be anticipated that the C-terminal region may be involved in folding in an active conformation of RBD. This assumption will be addressed by a detailed structure analysis of *Bst*L2 or its mutant desN in complex with the 23S rRNA fragment.

Acknowledgments

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