Activation of the RegB Endoribonuclease by the S1 Ribosomal Protein Is Due to Cooperation between the S1 Four C-terminal Modules in a Substrate-dependant Manner*

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The RegB protein, encoded by the T4 bacteriophage genome, is a ribonuclease involved in the inactivation of the phage early messenger RNAs. Its in vitro activity is very low but can be enhanced up to 100-fold in the presence of the ribosomal protein S1. The latter is made up of six repeats of a conserved module found in many other proteins of RNA metabolism. Considering the difference between its size (556 amino acids) and that of several RegB substrates (10 nucleotides), we wondered whether all six modules are necessary for RegB activation. We studied the influence of twelve S1 fragments on the cleavage efficiency of three short substrates. RegB activation requires the cooperation of different sets of modules depending on the substrates. Two RNAs are quite well cleaved in the presence of the fragment formed by the fourth and fifth modules, whereas the third requires the presence of the four C-terminal domains. However, NMR interaction experiments showed that, despite these differences, the interactions of the substrates with either the bi- or tetra-modules are similar, suggesting a common interaction surface. In the case of the tetra-module the interactions involve all four domains, raising the question of the spatial organization of this region.

The bacteriophage T4, like many others, takes advantage of host ribonucleases to mature and degrade its transfer and messenger RNAs (1, 2). However, T4 early transcripts are also inactivated by the phage-encoded RegB protein synthesized shortly after infection (3–6). The RegB protein is a ribonuclease that specifically cleaves in the middle of the tetra-nucleotide GGAG (3), with a strong bias toward the GGAG found in the Shine-Dalgarno sequence of the early messengers (6). In addition, RegB probably also cleaves essential host messengers. Indeed, it exerts a strong bacteriostatic/lethal effect on Escherichia coli cells (7). However, the in vitro activity of RegB is very low compared with its in vitro efficiency. In this context, of particular interest is the observation that the enzyme activity can be enhanced by a factor of up to 100 by the E. coli 30 S ribosomal subunit (9). In fact, the S1 protein alone is sufficient to achieve the same level of stimulation (9).

Working on the properties of RegB, we wished to analyze how S1 promotes RegB reaction. S1 is the largest E. coli ribosomal protein (for review, see Ref. 10). It is found in all Gram-negative and in several Gram-positive bacteria (11). A smaller form has also been described in plant chloroplasts (12). The protein plays an essential role in the initiation of translation (13). It is, in particular, required for the translation of messengers possessing weak (14) or no (15) Shine-Dalgarno consensus sequence. It is thus likely that it facilitates or even supplements the interactions between the Shine-Dalgarno region of the messenger and the 16 S ribosomal RNA. In addition, S1 is used by several phages. Besides its role in RegB activation, it is one of the four subunits of the replica of RNA bacteriophages (for review, see Ref. 16) and it interacts with the β-protein of phage λ (17). The fact that both its physiological function and its role in the RegB system involve the Shine-Dalgarno region suggests that S1 recognizes a similar signal in both cases. However, the nature of this signal is still an open question. The protein is able to bind poly(A), poly(U), and poly(C) oligonucleotides (10). Using a procedure based on UV cross-linking, the S1-binding site on Qβ phage mRNA was shown to contain a poly(U) stretch located upstream of the Shine-Dalgarno GGAG sequence (18), but other results indicate that S1 can also act as a poly(A)-binding protein (19). In agreement with these results, it has been proposed that S1 possesses two RNA-binding sites, one with better affinity for poly-pyrimidines, the others for poly-purines (19–22). The existence of two different RNA-binding sites may result from the fact that S1 consists of six repetitions of a conserved module found in many other proteins involved in RNA metabolism. The first two modules are responsible for the interactions of the protein with the ribosome, whereas the four C-terminal modules interact with mRNA (10). This latter region has been proposed to be elongated in shape, with a length of about 200 Å (10) but it fixes short RNA molecules of about 10 nucleotides. Similarly, S1 is able to accelerate the RegB-mediated cleavage of an unstructured decanucleotide (9) and of several structured molecules of about 20 nucleotides (23).

Considering the modular organization of S1, the discrepancy between the size of its RNA-binding region and that of several RegB targets, and the possible existence of two RNA-binding sites, we wondered whether all six modules were necessary for RegB activation. To approach this question, we cloned the sequences coding for twelve fragments of the S1 protein: the N-terminal bi-module, the C-terminal tetra-module, and the two three-modules, the three bi-modules, and the four mono-modules contained in the C-terminal region. The ability of all these fragments to enhance RegB cleavage was tested, and the interactions of two of them (the C-terminal tetra-module and the bi-module containing the fourth and fifth do-

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S1 Module Cooperativity in RegB Endoribonuclease Activation

MATERIALS AND METHODS

Cloning, Expression, and Purification of the S1 Protein and Modules—The S1 protein was purified from E. coli K12 strain ED8689 (M. Uzan, personal collection) by affinity chromatography on a poly(U) Sepharose 4B (Amersham Biosciences) column as described previously (10). It was stored at 4 °C in 10 mmol/l Tris-Cl (pH 7.4), 0.1 mmol/l EDTA, 50 mmol/l NaCl, and 1 mmol/l β-mercaptoethanol.

The DNA polynucleotides corresponding to the S1 fragments were PCR amplified from the strain used for S1 production and cloned in the vector pET-15b which contain the NdeI (5′) and BamHI (3′) sites, in phase with the 21-residue poly-histidine tag. The different S1 fragments were obtained by combining five 3′-oligonucleotides, corresponding to the beginning of the sequence (5′-F1), third (5′-F3), fifth (5′-F5), and sixth (5′-F6) module, and six 5′-oligonucleotides, corresponding to the end of the second (3′-F2), third (3′-F3), fourth (3′-F4), fifth (3′-F5), and sixth without the protein tail (3′-F6) and with the protein tail (3′-F6) modules. A NdeI restriction site was added to all 5′-oligonucleotides, an ochre (UAA) stop codon and a BamHI restriction site all to 3′-oligonucleotides: 5′-F1, GGCGATATGCTGAACTTCTCTG, 5′-F3, AAAAACATGAGAACCCGACAGATCAG, 5′-F4, GAAATCTCATGCTATGCTCTGCTG, 5′-F5, GGCGATATGCTGACCGAGTGTCGTCCG, 5′-F6, GAAATCCTATGGTTCCAAACAGGTTGC, 3′-F3, GCGGATCCGCGAGCGAAG, 3′-F4, GCGGATCCGCGAGCGAAG, 3′-F5, GCGGATCCGCGAGCGAAG, 3′-F6, GCGGATCCGCGAGCGAAG.

Ribonucleotide Synthesis and Purification—Ribonucleotides were synthesized chemically on a Amersham Biosciences LKB Gene Assembler Plus apparatus using Amersham Biosciences polystyrene substrates and purified using a Q-Sepharose high pressure liquid chromatography column running on a Beckman system. Prior to RNA loading, the column was equilibrated with 80% buffer A and 20% buffer B (buffer A, 1 mmol/l potassium phosphate (pH 7.0), 0.4 mmol/l-1 urea; buffer B, same as A with 1 mol-l-1 NaCl). All species could be separated using a linear gradient: 20% B to 80% B in 80 min.

Enzyme Assays and Kinetic Analysis—Cleavage reactions were performed in 10 mmol/l Tris-Cl (pH 8.0), 20 mmol/l NaCl, 1 mmol/l-1 dithiothreitol, and 0.1 mmol/l-1 EDTA at 37 °C. In case of the JR10 substrate (Fig. 1), 1 μm-l-1 3H-labeled RNA was incubated in the presence of 0.2 μm-l-1 RegB and 0.4 μm-l-1 S1 fragment in a final volume of 5 μl. 60 units of RNAse A were added to prevent the eventuality of parasite cleavage by RNAse A-like enzymes. In these conditions, the cleavage of the Selex12 and Selex26 molecules was too fast to be correctly analyzed. The amount of enzyme and of S1 fragment was, thus, reduced by a factor of 2. In parallel with each reaction, a control was performed by incubating the RNA in the same conditions (buffer, S1 fragment, and RNAse) without RegB. In addition, a ladder, obtained by partial digestion of the substrate, was always added to obtain a measurement of the product lengths. Aliquots (5 μl) were typically taken at 5, 10, 20, 30, 45, and 60 min for JR10 and 1, 3, 5, 10, and 20 min for the S1ex molecules. They were mixed with 3 μl of loading blue (8 mol-l-1 urea, 20 mmol-l-1 EDTA, 10% glycerol, and 0.05% bromphenol buffer), heated 2 min at 98 °C, and put on ice. The substrates and products were separated by gel electrophoresis (18% polyacrylamide, 8 mol-l-1 urea) and detected by autoradiography. The band intensities were quantified by phosphorimaging (Molecular Dynamics). Most reactions were carried out for two or three times.

NMR Experiments—All NMR experiments were performed on a Bruker DRX600 spectrometer equipped with a gradient triple resonance TCI X probe. The spectra were processed off-line using the GIPAF software (26) running under LINUX on a personal computer and analyzed using XEASY (27). The fast-HSQC sequence (28) was used for all experiments.

A first series of HSQC was realized on all fragments in a standard NMR buffer (20 mmol-l-1 phosphate (pH 6.3), 200 mmol-l-1 NaCl) at 30 °C. The three- and tetra-modules were also probed at 25 and 35 °C. To prepare the interaction experiments, a second series was also recorded in 50 mmol-l-1 Tris (pH 8.0), 20 mmol-l-1 NaCl, 4 mmol-l-1 β-mercaptoethanol at 30 and 35 °C for the F6short, F45, F3–5, and F3–6 fragments. In all cases, the concentration varied between 250 μmol-l-1 and 1 mmol-l-1.

Four series of titration experiments were carried out to analyze the interactions between the F45 and F3–6 bi- and tetra-modules and the JR10 and S1ex substrates. The F45/Sellex26 and F3–6/Sellex26 series were realized with two different RNA batches, and in the case of the F3–6/JR10, the HSQC were recorded at two temperatures (30 and 35 °C); all others were recorded at 30 °C. The protein was dialyzed against Tris (pH 8.0) and used at a concentration of 250 μmol-l-1. The corresponding amount of RNA was dispatched in four fractions and lyophilized after dialysis against pure water and pH neutralization. Each titration point was done by removing the NMR sample from the tube, mixing it with the lyophilized RNA and putting it back in the NMR tube. Five HSQC were thus recorded for each series, corresponding to RNA/protein ratios of 0.1, 0.25, 0.5, 0.75, and 1. All recording parameters were kept rigorously constant; the only modification concerned probe tuning and field shimming. The experiments were carried out with 200 rows of 512 points, each row corresponding to 128 scans. The spectral width was set to 22 (15N) and 5 (1H) ppm and the frequency offset to 7300 (15N) and 4800 (1H) Hz. The spectra were processed identically, to obtain 512 × 512 points real matrices. Small adjustments of the zero order phase in the w2 (1H) dimension, less than 5° were necessary to phase the matrices correctly. The difference maps were obtained by subtracting the reference matrix from the one of interest.

RESULTS

Choice and Production of the S1 Fragments and of the Substrates—The S1 protein is the largest E. coli ribosomal protein (556 amino acids in K12). It is composed of six repeats of a conserved domain, called the S1 domain, and is found in many other proteins involved in the RNA metabolism. Considering this, we wondered whether the six modules were necessary for RegB activation. The fragment consisting of the two N-terminal modules (F12) has been reported to be involved in the S1 interaction with the ribosome, whereas that encompassing the four C-terminal domains (F9–6) is responsible for RNA binding (10). We thus decided to isolate these two fragments together with F3–6 sub-fragments of the two former (F4–5 and F4–6), the three bi-modules (F34, F45, and F56) and the four mono-modules (F3, F4, F5, and F6) (Fig. 1). The module extremities were chosen according to the alignment published by Bycroft et al. (29). The linkers preceding and following each fragment were 2

The abbreviations used are: HSQC, heteronuclear single-quantum coherence; SAXS, small angle x-ray scattering experiment; NRE, nucleosibin recognition elements.
FIG. 1. Sequence of the S1 protein, fragments of the protein, and RNA substrates used in this study. The S1 domains are indicated in red in the sequence, the linkers in blue, and the residues conserved in at least three modules in green. The domain limits and the alignment are those proposed by Bycroft et al. (29). The same colors (red for the domains, blue for the linkers) have been used for the representation of the fragments. As indicated, all but the F6short include the linkers preceding and following the first and the last domain, respectively. The sixth module was produced twice: once with the C-terminal extension of the protein (F6long) and once without (F6short). The sequences of the three RNA substrates are represented with their predicted secondary structure, calculated with the mfold software (31). The GGAG cleavage site is indicated in red.

fragment were always included, and all fragments containing the sixth module but one were constructed with the C-terminal 30-amino acid tail. Indeed, the F6 domain was cloned twice, once with the C-terminal tail (F6long) and once without (F6short). All fragments were easily cloned in a pET-15b vector and expressed. All but the F5 were purified from the soluble fraction. The F5 fragment was produced as inclusion bodies but could be recovered by an unfolding-refolding procedure (see “Material and Methods”).

To compare the activity of all these fragments on RegB kinetics, we needed substrates whose cleavage was markedly accelerated by the whole protein. In addition, because one of our objectives was also to study the interactions between the substrates and the protein fragments by NMR, we wanted to use RNA molecules as short as possible. In their initial study of the role of S1 in RegB activation, Ruckman et al. (9) used a decanucleotide whose cleavage is very low in the absence of S1 but is accelerated by a factor of about 80 in its presence. This molecule (CUUUGGAGGG, hereafter designed JR10) clearly met our criteria. In addition, in a previous study (23), we looked at the influence of the substrate conformation on RegB cleavage efficiency. For this, we took advantage of a published study (30) carried out in the aim of selecting good RegB substrates, in the presence of S1, by the SELEX method, and we used several short fragments derived from one of the reported clones. Two of the molecules we analyzed at this occasion (Selex12 and Selex26) also suit our present requirements. As shown in Fig. 1, these two Selex molecules present strong similarities to each other and are very different from JR10. They are longer (20 nucleotides for Selex12 and 23 for Selex26). Their composition is strongly biased against U (only one each), whereas JR10 possesses five G, three U and only one A and one C. They present similar secondary structure as predicted by the mfold software (31), whereas JR10 seems to have no stable conformation.

Enzymatic Analysis of S1 Fragment Influence on RegB Activity—We first tested the effect of all S1 fragments on the Selex12 and Selex26 oligoribonucleotides by following the reaction kinetics for 20 min. The electrophoresis gels and kinetic curves corresponding to the cleavage by RegB alone or in the presence of all but the mono-modules are shown in Fig. 2. The data first show that there is, as expected, a marked difference between the cleavage ratio in the presence of S1 (nearly 100% after 20 min) and in its absence (less than 1%). They also demonstrate that the different fragments have variable effects on the reaction rate enhancement but have no influence on the position of the cutting event. Finally, they indicate that all fragments have similar effects on the Selex12 and Selex26 oligoribonucleotides.

As expected, the F3–6 (C-terminal) fragment induces the same enhancement as the whole S1 protein, whereas the F12 (N-terminal) has no influence on the cleavage rate. All other fragments can be classified in three groups. The F3–5 three-module has the same effect as S1 and F3–6. The F45 and F4–6 fragments also enhance the cleavage rate but to a lesser extent, with cleavage ratios about half that obtained using S1, F3–5, and F3–6 proteins. Finally, the F34 and F56 bi-modules and all mono-modules have no effect. Looking in detail at the kinetic curves, a difference appears between the two substrates. The cleavage is higher for the Selex12 substrate than for Selex26 in the presence of S1 and the F3–6, F3–5, and F4–6 fragments but is lower in the presence of the F45 bi-module. We previously observed (32) that Selex12 was cleaved better than Selex26 in the presence of S1. Interestingly, the ratio between the cleavage values of the two substrates is conserved (Selex12 cleavage/Selex26 cleavage ~1.2) for the four S1, F3–6, F3–5, and F4–6 proteins. The second observation is more puzzling, but it is difficult to determine whether it is really significant, considering that the data on the S1, F3–6, F3–5, and F4–6 kinetics came from one gel and that on F45 from another. In fact, the values obtained for RegB alone (Fig. 2, both gels) and in the presence of F12 (first gel) or of F34 and F56 (second gel) are also lower for the second gel than for the first.

In a second series of experiments, we looked at JR10 cleavage. We first notice that although S1 clearly enhances the cleavage of this substrate, the effect is less pronounced than for Selex26 and Selex12. We thus decided to follow the kinetics for 1 h in the presence of S1 and the F3–6, F3–5, and F4–6 fragments (Fig. 3, left gel). Interestingly, this experiment indicates that the C-terminal region (F3–6), as expected, is able to promote cleavage, but to a lesser extent than the whole S1 protein. It also shows that the F3–5 and F4–6 three-modules have little, if any, effect on cleavage efficiency. A second set of reactions was then performed using the bi-modules (F34, F45, and F56) and repeating the three-modules. Considering the projected slow rate of the reaction, only two points were recorded at 30 and 60 min. The cleavage was indeed very low, but we remark that after 30 min, the product ratio obtained in the
presence of the F3–5 and F4–6 three-modules (around 5%) is actually twice that obtained in the presence of the F34, F45, and F56 bi-module (around 2.4%), suggesting a slight effect of the former.

Choice of NMR Conditions—Two fragments of the S1 protein were of particular interest: the F3–6 C-terminal region and the F45 bi-module, which is the smallest fragment able to promote cleavage by RegB and is part of all active fragments. To try to understand the system better, we decided to analyze the interactions between these fragments and the substrates by nuclear magnetic resonance. More precisely, we decided to follow the effect of increasing amounts of RNA on the HSQC spectra of both fragments. The HSQC experiment correlates the chemical shifts of the backbone and side-chain 15N nucleus with those of their attached protons and can be considered a fingerprint of the backbone structure, because any structural perturbation will modify the spectrum.

Before doing the experiments, we needed to determine the best conditions. We first recorded an HSQC on all fragments using a standard NMR buffer (20 mmol/liter–1 phosphate, 200 mmol/liter–1 NaCl, 5 mmol/liter–1 β-mercaptoethanol) at pH 6.0. This pH was chosen because of the better quality of the NMR spectra at acidic pH because the exchange rate of the amide proton is slower. In all cases, the quality of the spectra is indeed very high (Fig. 4). The number of peaks is close to that expected. Even in the case of the F3–6 fragments, despite the crowding of the central region, more than 300 peaks, among 380, are readily identifiable. They are well defined, with similar intensities and their 1H frequencies are spread over nearly 4 ppm. This indicates that all fragments are correctly folded, the large 1H spectral width being in agreement with the module–expected high content in β-strands (29). The behavior of the F3–6 fragment, however, was puzzling. The spectrum previously discussed and reported in Fig. 4C was recorded at 35°C but below 30°C only a few peaks remained (Fig. 4D). By comparison with the spectra of the F6long and F6short fragments, the remaining peaks could be assigned to the 30-amino acid C-terminal tail of the protein, which was demonstrated, by the same, to be unstructured. The disappearance of the peaks below 30°C could be due either to the formation of a stable (and reversible) high molecular weight multimer or to the existence of an equilibrium in the intermediate exchange regime between two protein states (e.g. between two conformations or between a monomer and a dimer). To discriminate between these two possibilities, we recorded a small angle x-ray scattering experiment (SAXS) on a 250 μmol/liter–1 F3–6 sample in NMR buffer at 30 and 35°C. This experiment allows precise determination of the particle mean mass. We measured an identical value of 57.1 kDa at the two temperatures, compatible with a monomer-dimer (70/30%) equilibrium.

However, the NMR and SAXS experiments were recorded, as explained above, at pH 6.0, whereas the cleavage experiments were performed at pH 8.0. It was thus necessary to see whether the equilibrium had the same properties in both conditions. Accordingly, we recorded the NMR spectra of the F3–6 fragments and reran the SASX experiment in the cleavage buffer at
S1 protein, the two F3–5 and F4–6 fragments with JR10 and S26. Observation of the periphery of the F45/JR10 difference map. However, direct observation of the corresponding HSQC indicates that the two positive (blue circles) and four negative (red circles) peaks that are absent in the reference spectrum. These effects are not visible in the F45/JR10 difference map. However, direct observation of the corresponding HSQC indicates that the two positive (blue) peaks and, at least, two of the negative (red) peaks do actually appear in the presence of JR10 also. They are simply much fainter. This seems to be a general observation. In the presence of RNA many peaks shift (juxtaposition, on the difference map, of a blue and a red dot of similar intensity). As exemplified by the two effects indicated by arrows, the shifts are smaller in the presence of JR10 than in the presence of Selex26. Altogether,
FIG. 4. HSQC spectra recorded at pH 6.0 with F6short (A), F6long (B), F45 (C), F4–6 (D), F3–6 at 35 °C (E), and F3–6 at 25 °C (F). All spectra but that presented in E were recorded at 25 °C. The peaks present in the F6long spectrum and absent in F6short (and thus corresponding to the C-terminal extension) are indicated by small boxes in the F6long (B) spectrum. These peaks are more intense than the others, and the dispersion of their ¹H chemical shifts is small (less than 1 ppm), strongly indicating that the C-terminal tail is not structured. The same peaks are found in F (F3–6 at 25 °C), showing that the only part of the protein observable in these conditions is also its C-terminal tail.
these observations strongly suggest that the JR10 and Selex26 molecules both interact with the same area of the F45 fragment, but with different affinities.

The same analysis was carried out on the F3–6 tetra-module (Fig. 6). As previously indicated, the spectra are very crowded, and it is more difficult to observe directly the effects on the HSQC. However, the difference maps reveal that there are many, actually more than in case of the F45 bi-module. As previously, the two maps seem very similar at first sight, the main difference being again the intensity of the effects. However, the processes seem more complicated. A peak is indicated by an arrow on the reference HSQC. Strikingly, as shown in Fig. 6, its intensity increases in the presence of the Selex26 RNA, whereas it decreases slightly in the presence of JR10. Similarly, we also indicated another effect on the difference maps, very strong for the interaction with Selex26 but absent with JR10. This suggests that although many induced effects are identical (with different intensities) for the two RNA molecules, others are specific to Selex26. We also compared the two F3–6 difference maps with the HSQC spectra of the F45, F3–5, and F6short fragments recorded in the same conditions (Fig. 7). It is clear that many effects are not superimposed with the F45 peaks, indicating that they correspond to residues not belonging to this fragment. Indeed, most of these peaks are readily superimposed with peaks of either the F3–5 HSQC (in red on Fig. 7) or the F6short HSQC (in blue). Some remain unassigned. They may correspond to the C-terminal tail of the protein, or to peaks involved in an interface (whose chemical shifts would be different in the whole protein and in the fragments analyzed).

The use of HSQC to detect the effect of RNA addition on the F45 and F3–6 fragments focuses the analysis on the backbone of the proteins. Nevertheless, it also shows that several side-chains are involved in the complexes. The four peaks highlighted by red circles in Fig. 5 actually correspond to correlations between the N-H and H-H atoms of arginine side-chains. These peaks are negative because they are folded back in the spectra, their actual N frequencies (about 85 ppm) being outside of the spectral window analyzed. The green circles correspond to the disappearance of asparagine and/or glutamine NH side-chain correlations. Finally, the blue circles show the appearance of two peaks in an empty region of the reference spectra. The arrows indicate two peaks undergoing a marked shift in the presence of the RNAs. As discussed under “Results,” the shifts are larger for the interaction with the selex26 RNA molecule, suggesting a higher affinity of the protein for Selex26 than for JR10.
of them have an indirect origin such as an induced structural modification of the protein or a change in the amide proton exchange rate. The latter interpretation, in particular, is supported by the observation that the intensity of many peaks increases (blue dots in the difference maps) in the presence of RNA, suggesting a slower exchange rate.

**DISCUSSION**

As indicated in the introduction, the S1 protein is composed of six repeats of a conserved module of about 80 amino acids organized in a five-stranded β-barrel. The distance between the N and C termini of each module is ~30 Å (29), a value compatible with that deduced for the entire protein (between 230 and 280 Å, with an axial ratio of about 10) from hydrodynamic or x-ray scattering experiments (10). In contrast with this large size, the dimension of the RNA-binding site seems rather small. It has been evaluated by different techniques to correspond to unstructured fragments of about 10–12 nucleotides (10, 18, 19), with lengths of about 40 Å. The generation by the SELEX method of high affinity RNA ligands to S1 provided molecules consisting of a pseudoknot whose central helix was also formed by 10 nucleotides (33). The S1 protein is able to promote the cleavage of unstructured decanucleotides (e.g. JR10 used in this study). Considering the apparent discrepancy between the size of S1 and that of its RNA targets, we wondered whether all modules were necessary for a given biological activity or whether each has evolved to acquire specialized functions. A first answer to this question is provided by the observation that the first two modules are involved in ribosome binding, whereas the four following are responsible for the mRNA recognition, but the question remains open for the four C-terminal modules. We thus decided to analyze the ability of several fragments of the S1 protein, and in particular of all sub-fragments of the C-terminal region, to promote the RegB cleavage of several short RNAs.

The cleavage experiments first confirm that the ability of S1 to enhance the RegB rate is due to the C-terminal region of the protein (F3–6 fragment), but they also suggest that the removal of the N-terminal part alters its properties (as indicated by the comparison of the F3–6 and S1 efficiencies with JR10 as substrate). They also demonstrate that the different C-terminal modules are not equivalent. The F45 fragment is the only bi-module able to accelerate the cleavage reaction (using the Selex molecules), but to a lesser extent than the entire protein. The third module is always required and is sufficient in the case of the Selex substrates to compensate for the difference. The sixth module has no influence on the cleavage of the Selex molecules but is strictly required for JR10. These experiments also show that there is a strong cooperativity between the modules. The fourth and fifth modules are only active when associated in the F45 fragment, as shown by the absence of activity of the F4 and F5 mono-modules and of the F34 and F56 bi-modules. The third module is only active when associated with the fourth and fifth in the F3–5 fragment, as shown by the absence of activity of the F3 and F34 fragments. Finally, the sixth is only active when associated with the three others in the F3–6 fragment, as shown by the absence of activity of the
F6 and F56 fragments and by the very weak effect of the F4–6 three-module.

A second observation is that the efficiency of the fragments depends on the substrates. The F45 fragment is sufficient to partially activate the cleavage of the Selex molecules but not of JR10. The F3–5 tri-module is sufficient to reproduce the effect of the entire S1 protein on the two Selex molecules, but the complete C-terminal region (F3–6) is required to accelerate the cleavage of JR10. Interestingly, a similar property of the sixth module (indispensable for one activity, dispensable for another) has been recently reported in the initiation of translation (34). The authors showed that the S1 ssyF mutant codes for a protein lacking the sixth module (the last 92 residues of the protein, more precisely). They also showed that this mutation hardly affects the initiation of translation of the E. coli messenger RNA but abolishes the autorepression of S1 translation. This strongly suggests that the sixth module is indispensable for the down-regulation of the translation of S1 but dispensable for the initiation of translation. A related observation has also been made with the nucleolin protein (35). Nucleolin consists of four RNA binding domains of about 80 amino acids. It is able to bind two kinds of substrate: RNA fragments containing a short hairpin (NRE for nucleolin recognition elements) or an unstructured stretch of 11 conserved nucleotides (ECM for evolutionary conserved motif). Interestingly, the first two domains of nucleolin are necessary and sufficient for its specific interac-

![Figure 7: Identification of the module involved in the interactions between the F3–6 tetra-module and Selex26 or JR10.](https://example.com/fig7.jpg)
with the NRE hairpin, whereas all four domains are required for the binding of the unstructured ECM.

Several hypotheses can account for the above results. First, it was not excluded that some of them result from structural problems induced by the truncation of the protein in fragments (some mono- or bi-modules, for example, may lose their structure). Alternatively, we may speculate that S1 possesses different RNA-binding sub-sites, one recognized by the Selex molecules across the fourth and fifth modules, another binding JR10 and spanning the third and sixth modules. This hypothesis is supported by several studies suggesting the existence of two S1 polynucleotide-binding sites (19–22). However, we may also imagine that both molecules interacts with the F45 fragment but that JR10 needs more contacts (with the third and sixth modules) because its affinity is too low or because its interactions with the F45 fragment alone are insufficient to allow the proper orientation or conformation.

The NMR experiments first demonstrated that all fragments are structured and stable and that the modules have conserved their integrity in all of them. In addition, together with the SAXS experiments, they strongly support the existence of a monomer-dimer equilibrium of the F3–6 fragment at pH 6.0. At first sight, this provided an elegant model to explain our results, in particular the strong cooperativity between the third and sixth modules observed in JR10 cleavage. Indeed, even with the hypothesis of a completely extended organization of the fragment, it is possible to bring the third module of one molecule in close vicinity to the sixth of a second in a symmetric dimer. It thus seemed possible to propose that the Selex molecules interact with the fourth and fifth modules either only in the monomer or in both the monomer and the dimer states, whereas the JR10 would bind the third and sixth modules in the dimeric form only. However, the subsequent controls at pH 8.0 indicated that the F36 fragment is always monomeric in cleavage buffer and thus ruled out this model.

Actually, the NMR interaction experiments carried out at pH 8.0 show that the two substrates bind both the F45 and F3–6 fragments. In the case of the F45 fragment, the effects induced by the addition of both RNA seem similar. Their intensities, however, are different, suggesting that the ability of the F45 fragment to accelerate the cleavage of Selex26 but not of JR10 could be due to a stronger affinity of the protein for the first substrate. As for the F3–6 protein, most of the effects were observable in the two experiments, with the same bias in their intensities. However, we found at least two effects specific to interactions with Selex26. According to these results, the cleavage activation of the Selex and JR10 substrates obtained with different sets of modules would not result from interactions of the two RNAs with different sub-sites of the protein, but are more likely due to different use of the same interaction area. Interestingly, at the end of their study of nucleolin (35), the authors note that they “were unable to detect interactions of [the NRE and EMC] RNA with the same nucleolin molecule.” This suggests that the situation we encountered here (different action of the fragments toward the two families of substrate, but strong similarities between the effects induced by the interaction, suggesting a common interaction surface) may be shared by the two systems.

The comparison of the F36 difference map with the HSQC spectra of the F45, F3–5 and F6 short proteins clearly shows that the binding of both RNAs on the F3–6 fragment induces modifications not only on the fourth and fifth modules but also on the third and sixth. This was not unexpected in the case of the JR10 substrate but it is more surprising with the Selex26 RNA. Again, this observation raises the problem of S1 spatial organization. First, we have to point out that the technique we used cannot distinguish between direct contact and indirect modification of the protein structure. But, considering the large number of effects observed, it is unlikely that they all correspond to direct contacts. It is thus very likely that the binding of the substrates to the F3–6 fragment induces structural perturbations of the protein. Under these conditions, it is not impossible that there is no direct interaction between the RNA and the third and sixth modules, but merely coupling between the substrate binding and a perturbation of the structure and/or positioning of the two modules. Obviously, with this hypothesis, the fact that the third and sixth modules are necessary for complete activation of the reaction implies that the coupling acts in both directions: i.e. that it is necessary to allow the correct substrates binding. However, this is not the only possibility. A comparison was made between the cryo-electron microscope map of a S1 containing E. coli ribosome and an x-ray crystallographic structure of the 30 S sub-unit lacking S1 (36). The deduced shape of the S1 protein is complex, with a central globular portion and two stretched arms, very different from the tri-axial body proposed in view of the SAXS experiments. In addition, the S1 protein possesses two cysteines, both in the fourth module. One (Cys-349) is exposed in the native protein but is protected in the presence of RNA (8). The other (Cys-292) is protected in the entire protein but is accessible in the MIS1 truncated form of S1 lacking the sixth module (10). This suggests a structural relationship between the fourth and the sixth modules. It thus seems possible to imagine that the four C-terminal modules of S1 are actually able to interact simultaneously with short RNA substrates.

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Activation of the RegB Endoribonuclease by the S1 Ribosomal Protein Is Due to Cooperation between the S1 Four C-terminal Modules in a Substrate-dependant Manner

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