

Formation of SRP-like particle induces a conformational change in *E. coli* 4.5S RNA

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Abstract

E. coli P48 protein is homologous to the SRP54 component of the eukaryotic signal recognition particle. In vivo, P48 is associated with 4.5S RNA which shares a homology with eukaryotic SRP RNA. To study the interaction between P48 and 4.5S RNA in vitro, we used 4.5S RNA with fluorescein coupled to the 3'-terminal ribose. Upon binding of P48, the fluorescent 4.5S RNA shows a substantial decrease in fluorescence. Fluorescence quenching as well as anisotropy measurements reveal that the effect is not due to a direct interaction of P48 with the dye. This suggests that the binding of P48 induces a conformational change in 4.5S RNA which affects the structure at the 3' end of the RNA. From equilibrium titrations with fluorescent 4.5S RNA, a dissociation constant of 0.15 μM is obtained for the RNA · protein complex. The formation of the complex is not affected by GTP binding to or hydrolysis by P48.

Key words: 4.5S RNA; Signal recognition particle; P48 (FFh) protein; Ribonucleoprotein; Fluorescence labeling; *E. coli*

1. Introduction

The P48 protein from *E. coli* (also termed Fth protein) is known to exist in a ribonucleoprotein complex with 4.5S RNA that resembles part of the mammalian signal recognition particle (SRP) [1,2]. P48 is homologous to the SRP54 subunit of SRP [3,4], and 4.5S RNA, at the apex of the molecule, harbours a domain which is homologous to domain IV of SRP RNA [5,6]. Mammalian SRP consists of six polypeptides [9, 14, 19, 54, 68, 72 kDa] and a 7S RNA of ~ 300 nt [7]. The SRP54 subunit of SRP interacts with the signal sequence of secretory proteins as it emerges from the ribosome, and the SRP-ribosome complex is then targeted to the ER membrane where translocation of the presecretory protein occurs [8–11].

For the *E. coli* P48-4.5S RNA complex, crosslinking data show that it binds to ribosomes which expose the signal sequence of presecretory proteins, and it is likely that the P48-4.5S RNA complex acts as a chaperone that keeps the presecretory protein in a translocation-competent conformation [12]. P48 and 4.5S RNA are both essential for cell growth [13,14]. However, whereas depletion of P48 leads to serious deficiency in protein secretion [14], a depletion of 4.5S RNA or a defect in 4.5S RNA has no general influence on protein secretion in *E. coli* but leads to the induction of the heat shock response [2,15].

Homologs of 4.5S RNA have been found in a number

of bacteria and all have a conserved apical stem-loop domain in common with mammalian SRP RNA [16]. A complex homologous to the P48-4.5S RNA complex of *E. coli* has been shown in *Mycoplasma mycoides*, and the protein binding site on the RNA has been located in the apical stem-loop structure [17]. For *E. coli* 4.5S RNA, it has been shown that several nucleotides located in the conserved domain of 4.5S RNA are essential for the binding of P48 [18].

The sequences of SRP54 and the bacterial homologs contain a GTP binding domain in the N-terminal part of the protein [3,4]. In contrast to mammalian SRP54, the bacterial homologs in *E. coli* and *Mycoplasma mycoides* exhibit an intrinsic GTPase activity [19,20].

In the present contribution, we have studied the interaction of P48 with 4.5S RNA. We show that 4.5S RNA labeled with fluorescein at the 3' end exhibits a fluorescence change upon binding of P48, thus indicating a conformational change of the RNA.

2. Materials and methods

2.1. Preparation of 4.5S RNA

4.5S RNA was isolated from the overexpressing strain *E. coli* Hb 101 harbouring the plasmid pSB 832 [21]. Total nucleic acids were obtained from late-log cells by phenol extraction, and high molecular weight RNA was precipitated by adding 1 volume 8 M LiCl and centrifuging for 2 h at 0°C. The supernatant was applied to gel filtration on Superose 6 prep grade (Pharmacia) (1.6 × 50 cm) in buffer A (10 mM Bis-Tris-HCl, pH 6.0, 10 mM MgCl₂, 1 mM EDTA) containing 0.1 M LiCl in addition. The final purification of 4.5S RNA was performed by FPLC on a MonoQ anion-exchange column (HR5/5, Pharmacia) run at room temperature at a flow rate of 0.5 ml/min using a 20-ml linear gradient between 0.45 and 0.55 M LiCl in buffer A. Fractions containing 4.5S RNA were pooled, and the RNA was precipitated by ethanol. The pellets were dissolved in deionized water and stored at -20°C. The

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Abbreviations: SRP, signal recognition particle; 4.5S RNA_F, fluorescein labeled 4.5S RNA.

purified 4.5S RNA appeared as a single band on polyacrylamide gel electrophoresis with and without urea. Concentrations of 4.5S RNA were determined on the basis of an extinction coefficient of $9.0 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 260 nm, as estimated from the known extinction coefficients of tRNA (76 nt) and 5S RNA (120 nt).

For radioactive labeling, 4.5S RNA was dephosphorylated with alkaline phosphatase and then labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (5000 Ci/mol; Amersham) using T4 polynucleotide kinase. Unincorporated label was removed by centrifugation through G-25 spun columns.

2.2. Fluorescence labeling of 4.5S RNA

4.5S RNA (100 A_{260} units/ml) was oxidized by incubation in 1 M KIO_4 , 0.1 M sodium acetate, pH 5.3, for 30 min at 0°C in the dark. The reaction was stopped by adding ethylene glycol to a concentration of 10 mM and incubating further for 5 min at 0°C . After ethanol precipitation the RNA was dissolved (550 A_{260} units/ml) in 0.1 M sodium acetate, pH 5.3, and reacted with 10 mM fluorescein-5-thiosemicarbazide (Molecular Probes) for 16 h at 0°C in the dark. The RNA was then ethanol-precipitated three times to remove unreacted dye and finally dissolved in buffer B (20 mM HEPES pH 7.5, 10.5 mM magnesium acetate, 0.5 mM EDTA, 100 mM NH_4Cl). Fluorescein-labeled 4.5S RNA (termed 4.5S RNA_F) was separated from unmodified RNA by FPLC on MonoQ using a gradient from 0.5 to 0.8 M LiCl in buffer B. Fractions containing 4.5S RNA_F were pooled and the RNA precipitated with ethanol. The pellets were dissolved in buffer B and stored in aliquots at -80°C .

The stability against hydrolytic loss of the dye from 4.5S RNA_F was evaluated by chromatography on MonoQ after incubation at 25°C for 60 min under the conditions of the spectroscopic measurements (buffer B). The extent of hydrolysis was less than 4%.

2.3. Fluorescence measurements

Fluorescence was measured in 4 mm \times 4 mm (i.d.) quartz cuvettes on a Schoeffel RRS 1000 spectrofluorimeter. Fluorescein fluorescence was excited at 470 nm and monitored at 515 nm. Ethidium fluorescence was excited at 475 nm and measured at 594 nm. Fluorescence anisotropy was measured as described [22]. The sample temperature was maintained at 25°C .

Titrations of 4.5S RNA_F with P48 were carried out at 25°C with an initial volume of 300 μl of 0.05 μM 4.5S RNA_F in buffer B. Buffer blanks of fluorescein-free samples were less than 2% of the signal with 4.5S RNA_F and were subtracted throughout. To correct for dilution during titrations, the data were normalized with data obtained from a titration with buffer alone.

The fluorescence anisotropy of 4.5S RNA-bound ethidium was measured in buffer B containing 0.5 M K acetate. To ensure the binding of ethidium (0.25 μM), an eightfold excess of 4.5S RNA (2 μM) was added.

Fluorescence quenching titrations were performed by measuring the fluorescence intensity in the absence (I_0) and presence (I) of increasing amounts of KI. To determine the quenching constant, K_{SV} , the data were analysed according to the equation $I_0/I = 1 + K_{\text{SV}}[Q]$, as described [22].

2.4. Construction of the P48 expression plasmid

The expression vector pDS12-48His6 was used for the expression of P48 extended by six histidines at the C terminus. The oligo-histidine tag allows a rapid purification of the protein by metal chelate chromatography [23]. pDS12-48His6 is derived from pDS12-48 [1] by introducing a *Bg*III site and nucleotides coding for six histidine residues at the C terminus of P48:

wildtype P48	His tail
gly phe pro gly arg	arg ser his his his his his his
GGC TTC CCT GGT CGC	AGA TCT CAT CAC CAT CAC CAT CAC TAA
	<i>Bg</i> III

2.5. Expression and purification of recombinant P48 protein

E. coli JM109 harboring the lac repressor plasmid pDMI,1 was transformed with pDS12-48His6. An overnight culture (10 ml) was

grown in LB medium with 100 $\mu\text{g/ml}$ ampicillin and 30 $\mu\text{g/ml}$ kanamycin and used to inoculate a 1-liter culture in the same medium. Cells were grown at 37°C until an optical density of 0.6 was reached. Then 2 mM IPTG was added and growth was allowed to continue for 2 h. The harvested cells were stored at -80°C .

The protein purification was carried out at 4°C . Cells were suspended in 6 volumes of buffer C (50 mM NaPO_4 , pH 7.8, 300 mM NaCl, 10 mM β -mercaptoethanol, 0.05 mM Pefabloc (Merck)) to which 1% Brij58 (Pierce) was added to improve the yield of extracted protein. After adding lysozyme (1 mg/ml), the suspension was stirred on ice for 1 h and then treated with DNase I (10 $\mu\text{g/ml}$) for 15 min. The suspension was then sonicated for 2 min on ice. After centrifugation for 1 h at 20,000 rpm, the supernatant was applied to metal chelate chromatography on Ni^{2+} -NTA-Agarose (Diagen) [23]. The chromatography was performed as described by the supplier. The supernatant was added to a 50% slurry of Ni^{2+} -NTA resin in buffer C including 10% glycerol and stirred on ice for 1 h. The resin was filled into a column and washed first with buffer C and then with buffer C containing 500 mM NaCl, 10% glycerol and 40 mM imidazol. P48 was eluted by applying a gradient up to 150 mM imidazol. Fractions containing P48 were concentrated with centricon-30 microconcentrators (Amicon), if necessary. The purity of the protein after Ni^{2+} -NTA-Agarose chromatography was more than 95% as judged by SDS gel electrophoresis. After overnight dialysis against buffer B containing buffer 1 mM DTE, 40% glycerol the protein was stored at -20°C . Protein concentrations were determined by the Bradford method using BSA as standard.

2.6. Gel electrophoresis

The gel mobility shift assay was performed on non-denaturing polyacrylamide gels as follows (N. Bilgin and M. Ehrenberg, personal communication). The electrophoresis buffer contained 50 mM Tris acetate, 75 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 6.5. A 7% polyacrylamide gel (70 \times 70 \times 1 mm) containing electrophoresis buffer was poured and run at 80 V in the cold room (4°C) until the Bromphenol blue had reached the bottom of the gel. The buffer was exchanged every 30 min. Fluorescence was made visible by UV light (302 nm). The gel was first stained with ethidium bromide and subsequently with Coomassie R-250.

3. Results

3.1. Preparation of fluorescent 4.5S RNA

Purified 4.5S RNA was oxidized at its 3'-terminal ribose using periodate and then reacted with fluoresceinthiosemicarbazide to yield a fluorescent 4.5S RNA, termed 4.5S RNA_F . 4.5S RNA_F could be separated from unlabeled 4.5S RNA by ion exchange chromatography (Fig. 1). The unmodified RNA elutes at 0.54 M LiCl whereas the 4.5S RNA_F elutes at higher salt concentration in two peaks (Fig. 1). For use in spectral measurements the two fluorescent peaks were pooled. Both exhibit the same ratio of fluorescence to A_{260} , which indicates that the stoichiometry of dye to RNA is the same for both peaks. Since the oxidation of the RNA creates two aldehyde groups at the 3'-terminal ribose, one or two dyes can be attached to the RNA. To determine the stoichiometry of dye to RNA for 4.5S RNA_F , we have measured the ratio of the absorbances due to the fluorescein dye (at 492 nm, $\epsilon \cong 6.0 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 7.5) and to the RNA (at 260 nm, $\epsilon \cong 9.0 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The measured ratio (0.062) corresponds to the ratio of the extinction coefficients (0.067), indicating that one dye is incorporated into the 4.5S RNA_F . Upon gel electrophoresis in the presence of urea, the 4.5S RNA_F runs as

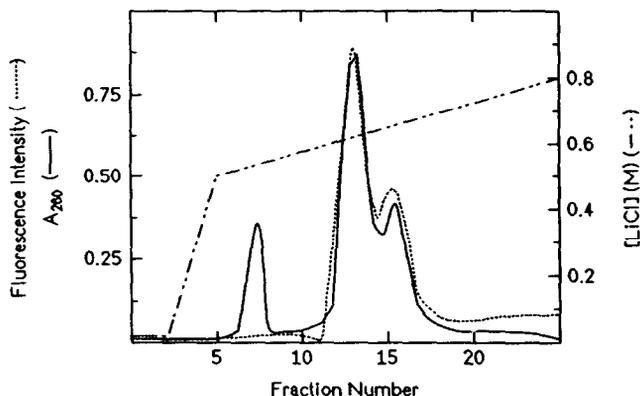


Fig. 1. Purification of 4.5S RNA_F by column chromatography. 4.5S RNA was labeled with fluorescein and then subjected to chromatography on MonoQ as described in section 2. Fractions 7–8 contain unmodified 4.5S RNA, while fractions 12–17 contain 4.5S RNA_F; the latter were pooled. The free fluorescein dye eluted only after addition of ethanol to the column (not shown). Absorbance and fluorescence were measured by flow-through detectors. Fractions of 1 ml were collected.

a homogeneous fluorescent band at the position of the unlabeled RNA (not shown). Thus, the heterogeneity of 4.5S RNA_F observed in the ion exchange chromatography (Fig. 1) is probably due to the presence of steric isomers of the dye at the 3'-terminal ribose.

3.2. Binding of 4.5S RNA to P48

First, we measured the formation of the complex between P48 and 4.5S RNA by filter binding. As shown in Fig. 2, virtually all 4.5S RNA is bound. From the concentration of P48 at which half-maximal binding is reached, a K_d of $\leq 0.3 \mu\text{M}$ is estimated.

The binding of P48 to 4.5S RNA and 4.5S RNA_F is also demonstrated by gel retardation (Fig. 3). In a non-denaturing gel, the complex containing the RNA migrates as a distinct band which is much slower than the free 4.5S RNA. The presence of P48 in the complex was determined by Coomassie staining (not shown). Free P48 due to its positive charge does not enter the gel. Since all 4.5S RNA_F is shifted at a protein to RNA ratio of 1:1, we conclude that the P48-4.5S RNA complex is a 1:1 complex and that both, the RNA and the protein are fully active in binding to each other. The lower staining intensity of the complex compared to the free RNA probably is due to interference with ethidium binding by the protein. The decrease of the amount of complex observed with an excess of protein most likely reflects the formation of a higher-order complex which does not enter the gel.

3.3. Binding of P48 changes the fluorescence of 4.5S RNA_F

The fluorescence properties of 4.5S RNA_F and of the 4.5S RNA_F:P48 complex are summarized in Table 1. The formation of the complex results in a fluorescence de-

crease of 30%, while the wavelength of the emission maximum is not changed upon binding. To show the specificity of the effect, we tested other proteins like elongation factor G (known to interact with 23S rRNA) and BSA. These proteins had no influence on the fluorescence properties of 4.5S RNA_F. If the fluorescence decrease were due to some direct interaction of the dye with the protein, then the accessibility of the dye for collisional quenching by iodide ions should be decreased. However, by measuring the fluorescence at increasing concentrations of iodide, we find that the quenching constant, K_{SV} , which is a measure for the accessibility of the dye for iodide ions present in solution, is increased rather than decreased upon formation of the complex (Table 1).

Finally, the freedom of rotational motion of the label was assessed by measuring the fluorescence anisotropy of the fluorescein label in free and P48-bound 4.5S RNA_F. The same low value was found in both cases (Table 1), which indicates that the dye has a high degree of rotational freedom both in the free RNA and in the complex.

Taken together, the unchanged emission wavelength, the increase of the quenching constant, and the unchanged mobility of the fluorescein label strongly argue against a direct contact of P48 with the label in the complex with 4.5S RNA_F. This is in good agreement with the results of footprinting [17] and site directed mutagenesis [18] experiments, which locate the P48 binding domain to the conserved apical stem-loop structure, distal from the 3' end of 4.5S RNA. As a consequence, the fluorescence decrease caused by the binding most likely is reflecting a conformational change at the 3' end of the RNA.

The anisotropy of 4.5S RNA_F is very low if compared with values reported for fluorescence labeled tRNA [22]. To answer the question whether the low anisotropy is due to a high mobility of the whole RNA molecule or it

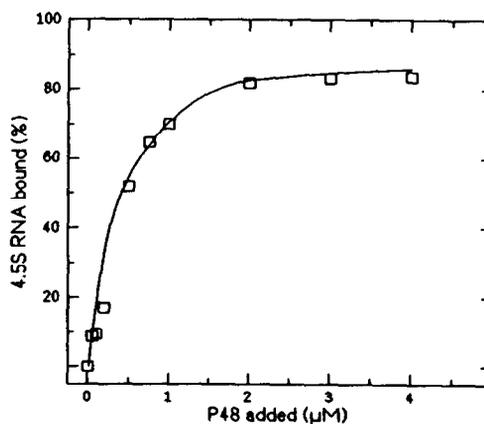


Fig. 2. Retention of the P48-4.5S RNA complex on nitrocellulose filters. 5'-[³²P]4.5S RNA (0.18 nM, 3×10^5 cpm/pmol) was incubated with P48 in 100 μl of 50 mM Tris-HCl, pH 7.5, 160 mM KCl, 90 mM NH₄Cl, 10 mM magnesium acetate for 10 min. The sample was filtered through a nitrocellulose filter and washed with 1 ml of buffer. The binding is given relative to the input of [³²P]4.5S RNA measured by TCA precipitation.

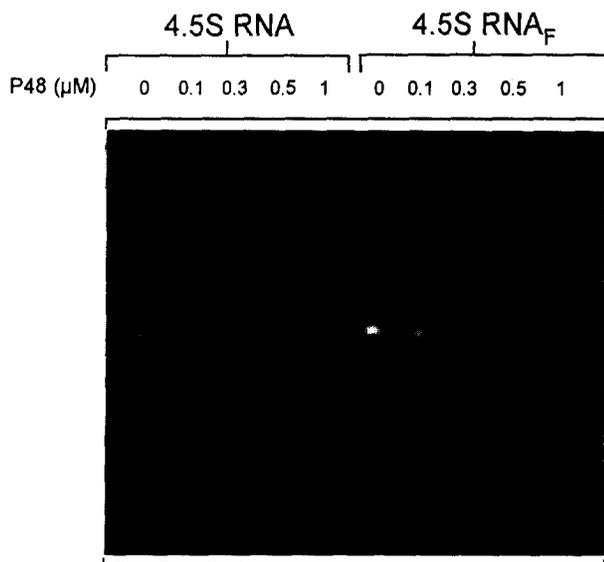


Fig. 3. Complex formation of 4.5S RNA and 4.5S RNA_F with P48. Samples containing 0.5 μM of 4.5S RNA and 4.5S RNA_F, respectively, were incubated with the indicated amounts of P48 in buffer B for 20 min at 25°C. After adding Ficoll to a final concentration of 2.5% (v/w) the samples (18 μl) were subjected to non-denaturing polyacrylamide gel electrophoresis as described in section 2. The gel stained with ethidiumbromide is shown.

is reflecting the mobility of the single-stranded 3' end of 4.5S RNA, the anisotropy of ethidium intercalated into 4.5S RNA was measured (section 2). The anisotropy of the fluorescence of the intercalated dye was found to be 0.17, not much lower than the values for ethidium labeled tRNA [24]. Since the anisotropy measured with intercalated ethidium is reflecting the mobility of the 4.5S RNA molecule, which appears to be rather low, the low value found for 4.5S RNA_F suggests that the fluorescein bound to the 3' end has a high rotational mobility relative to the RNA molecule. The mobility is probably due to the flexibility of the single-stranded 3' terminus of the 4.5S RNA and the flexibility of the linker through which the dye is attached.

3.4. Affinity of P48 for 4.5S RNA_F

The decrease in fluorescence emission of 4.5S RNA_F upon binding of P48 was used to determine the affinity of P48 for 4.5S RNA_F. In titration experiments, the fluorescence of 4.5S RNA_F was measured as a function of P48 concentration (Fig. 4a). The K_d calculated from the average of eight separate titrations was $0.15 \pm 0.05 \mu\text{M}$ (Fig. 4b). The binding is not affected by the presence of GTP. The specificity of the interaction is shown by the fact that the addition of a 50-fold molar excess of tRNA^{Phe} does not influence the fluorescence change. In contrast, the presence of unmodified 4.5S RNA in 10-fold molar excess significantly reduces the fluorescence effect (Fig. 4b). Comparison of the K_d values of the unmodified 4.5S RNA obtained by filter binding (0.3

μM) and of 4.5S RNA_F obtained from fluorescence titration ($0.15 \mu\text{M}$) reveals a slightly higher affinity of 4.5S RNA_F for P48. This is in agreement with the results of the gel retardation analysis (Fig. 4) where 4.5S RNA_F is also somewhat more active in complex formation than unmodified RNA.

4. Discussion

Recent data strongly suggest that P48 and 4.5S RNA, which both are essential for growth in *E. coli* [13,14], form a complex [1,2]. Most significantly, it has been demonstrated that the P48-4.5S RNA complex recognizes the signal sequences of presecretory proteins [12] and that depletion of P48 impairs protein export [14]. The interaction of P48 with 4.5S RNA involves the apical stem-loop structure which has a high degree of homology both in sequence and in secondary structure with domain IV of eukaryotic SRP RNA as well as with other bacterial 4.5S RNAs [5,6]. The related SRP-like RNP from *Mycoplasma mycoides* has been characterized recently [17,19].

The present study has been performed with 4.5S RNA and P48 obtained by overexpression in *E. coli*. Both preparations used were fully competent for complex formation as demonstrated by gel retardation. This experiment also reveals that the fluorescein-labeled 4.5S RNA_F is as active in forming the complex with P48 as the unmodified RNA. The dissociation constant of the complex ($0.15 \mu\text{M}$) indicates that the complex prevails in vivo, since both components are probably present in the *E. coli* cell in μM concentrations. It is to be noted that the formation of the complex does not seem to be affected significantly by the binding of guanine nucleotides to P48. Vice versa, the GTPase activity and the guanine nucleotide binding properties of P48 are not influenced by the presence of 4.5S RNA (data not shown).

As revealed by the fluorescence of 4.5S RNA_F, the binding of P48 induces a conformational change around the fluorescein at the 3' end of the RNA. On the basis of both the increased, rather than decreased, accessibility

Table 1
Properties of 4.5S RNA_F fluorescence in the absence and presence of P48

	Rel. fluorescence	K_{SV} (M^{-1})	Anisotropy
4.5S RNA _F	= 1.00	8.3 ± 0.4	0.05 ± 0.01
4.5S RNA _F + P48	0.70 ± 0.05	10.6 ± 0.3	0.05 ± 0.01

Data were obtained at 25°C in buffer B. Measurements were performed with $0.05 \mu\text{M}$ 4.5S RNA_F alone or in the presence of $4 \mu\text{M}$ P48. K_{SV} , Stern-Volmer quenching constant.

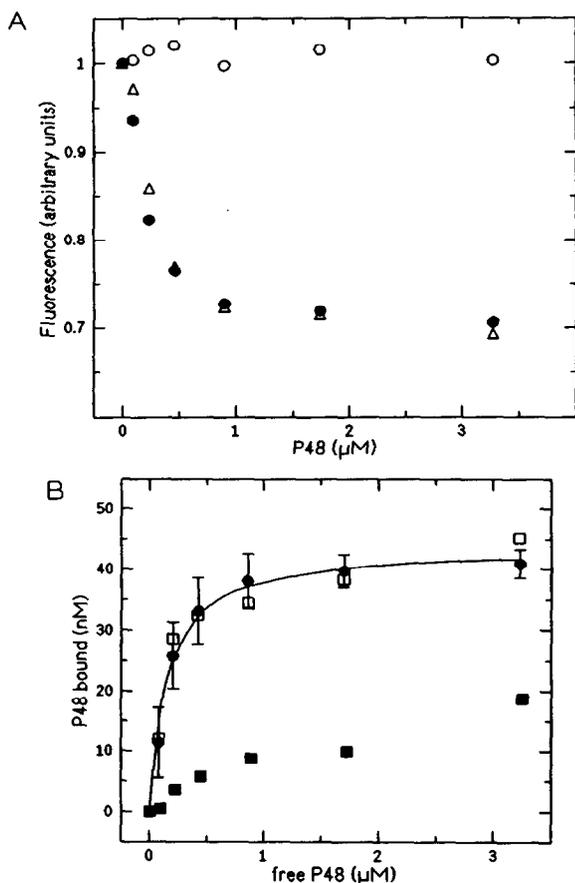


Fig. 4. Fluorescence titration of 4.5S RNA_F with P48. (A) Fluorescence titration. 4.5S RNA_F (50 nM) was titrated with buffer alone (open circles), with P48 (closed circles), or with P48 in the presence of 1 mM GTP (triangles). (B) Titration curves. The concentration of bound P48 was determined from the magnitude of the fluorescence change assuming a binding stoichiometry of 1:1. $K_d = 0.15 \mu\text{M}$ is obtained by nonlinear regression (continuous line) of the data obtained without competitor (closed circles; average of eight titrations, standard deviations indicated by bars). Also shown are titration curves obtained in the presence of a 50-fold molar excess of tRNA^{Phe} (open squares) or of a 10-fold excess of unlabeled 4.5S RNA (closed squares).

for quenching by iodide ions and the high rotational mobility retained by the dye in the complex, an interaction of the protein with the dye is rather unlikely. This is in keeping with the RNase footprinting data obtained for the homologous complex from *Mycoplasma* which also suggests that the protein does not cover the terminal double-stranded region of the even shorter RNA [17]. Thus, in order to explain the fluorescence decrease observed upon binding of P48 to 4.5S RNA_F, we suggest that, in the complex, the RNA adopts a conformation which differs from the one of the free molecule. The conformational transition which is primarily induced at the P48 binding site is transmitted to the 3' terminus of the RNA in an allosteric fashion and results in a more exposed position of the fluorescein label.

The presumed conformational change of the 4.5S RNA may be related to a conformational transition re-

ported for 7S RNA upon formation of the SRP [25]. The authors propose that, in the free RNA, four contiguous base pairs formed between distant parts of the 7S RNA constitute an important element of the three-dimensional structure of the molecule. An analogous interaction is possible in 4.5S RNA by pairing bases 12–14 with bases 53–51; the potential to form those base pairs is conserved among SRP RNAs [25]. The formation of base pairs between these regions would lead to a folded structure of 4.5S RNA which should differ in hydrodynamic properties from the extended structure suggested by the secondary structure model. The prevalence in solution of the latter structure is suggested by the results of laser light scattering measurements [26]. On the other hand, we find a fluorescence anisotropy for ethidium bound to 4.5S RNA, 0.17, which is close to the value observed for ethidium covalently attached to tRNA [24], thus suggesting a rotational motion of 4.5S RNA similar to tRNA which has a folded structure. The existence of two forms of 4.5S RNA with different electrophoretic mobility in polyacrylamide gels under low salt conditions (unpublished results) shows the principal ability of 4.5S RNA to adopt alternative conformations. While these findings indicate a higher order structure for free 4.5S RNA, more extensive studies are required to substantiate this contention.

The present observation of a conformational transition of 4.5S RNA induced by binding of P48 is of interest for the function of the complex. Allosterically induced long-range conformational changes mediated by the RNA component have been described for other RNPs such as ribosomes and spliceosomes [27,28]. The ability to undergo a conformational change in an all-or-none fashion ('switch') may be an important reason for the presence of the RNA component in these systems. Alternatively, the free 4.5S RNA in one conformation may exert a function that is different from the SRP function in the P48-bound conformation.

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