Binding of Escherichia coli ribosomal proteins to 23S RNA under reconstitution conditions for the 50S subunit

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ABSTRACT

    The RNA binding capacity of 50S proteins from E. coli ribosomes has been tested under improved conditions; purified proteins active in reconstitution assays were used, and the binding was studied under the conditions of the total reconstitution procedure for the 50S subunit. The results are: 1) Interaction of 23S RNA was found with 17 proteins, namely L1, L2, L3, L4, L7/L12, L9, L10, L11, L15, L16, L17, L18, L20, L22, L23, L24 and L29. 2) The proteins L1, L2, L3, L4, L9, L23 and L24 bound to 23S RNA at a level of about one copy per RNA molecule, whereas L20 could bind more than one copy (no saturation was observed at 1.8 copies per 23S RNA), and the other proteins bound 0.2 - 0.6 copies per RNA. 3) L1, L3, L7/L12 showed a slight binding to 16S RNA, L26 (identical with S20) strong binding to 16S RNA. 4) The binding of L2, L7/L12, L10, L11, L15, L16 and L18 was preparation sensitive, i.e. the binding ability changed notably from preparation to preparation. 5) All proteins bound equally well to 23S RNA in presence of 4 and 20 mM Mg^{2+}, respectively, except L2, L3, L4, L7/L12, L9, L10, L15, L16 and L18, which bound less strongly at 20 mM than at 4 mM Mg^{2+}.

INTRODUCTION

    Ribosomal proteins which can bind to their respective rRNA independently of other proteins are referred to as "binding proteins". Eleven proteins (L1, L2, L3, L4, L6, L13, L16, possibly L17, L20, L23 and L24) have been reported as binding proteins (see ref. '1 for review). Recently, additional proteins (L11 and L15) purified by a gentle isolation technique (2) were identified as binding proteins (3).

    The most searching criterion for an active ribosomal protein is its activity in reconstitution assays. In this paper we test 50S ribosomal proteins which are active in reconstitution assays for their ability to bind to 23S RNA. Notable differences from

the previous group of binding proteins are described.

MATERIALS AND METHODS

Ribosomes, ribosomal subunits, total proteins from the 50S subunit (TP50), and (23S+5S)RNA were isolated from E. coli K12, strain A19, as described (4). The separation of 23S and 5S RNA was reported previously (5). The method for two-dimensional gel electrophoresis followed ref. 6 with the modifications describ- ed in ref. 7. The intactness of the 23S RNA was analysed by poly- acrylamide gel electrophoresis as reported (8), except that the gels were scanned at 260 nm instead of being stained; after electrophoresis the gels were removed from the glass tube, plac- ed in a quartz cuvette, covered with glass distilled and degased water and scanned using a Gilford spectrophotometer 250 at 260 nm. The isolation of the ribosomal proteins will be described else- where (G. Wystup, H. Teraoka, H. Schulze, H. Hampl and K. H. Nierhaus, manuscript submitted).

The activity of the isolated proteins analysed in various reconstitution assays which have been used for studying the 50S assembly (9), the binding of [^{14}C]erythromycin (10), and the importance of the various proteins for peptidyltransferase activity (H. Hampl, H. Schulze and K.H. Nierhaus, manuscript in preparation).

Assay for RNA binding

7.5 A_{260nm} units of 23S RNA freed of 5S RNA, or 3.8 A_{260nm} units of 16S RNA, were incubated with 15 e.u. (equivalent unit; one equivalent unit is the amount of protein present in 1 A_{260nm} unit of 50S subunits) of an isolated protein under the condi- tions of the first or the second step of the two-step reconsti- tution procedure (20 mM Tris-HCl, pH 7.5, 4 mM Mg^{2+}, 400 mM NH_4^+ and 20 mM Tris-HCl, pH 7.5, 20 mM Mg^{2+}, 400 mM NH_4^+, res- pectively, see ref. 11). The total volume was 200 µl, which was incubated for 20 min at 44°C (first step) or 90 min at 50°C (second step). Samples containing 16S RNA were assayed only under the conditions of the first step.

After incubation the sample was layered onto a linear

sucrose gradient (4 ml; 5-20%) containing the same ionic con-
centrations as the sample, and was centrifuged for 90 min at
260.000 x g. The gradient was fractionated and the absorption
monitored at 280 nm. The fractions comprising the shoulder on
the fast-running side of the RNA peak were pooled, 50% trichlo-
roacetic acid was added ($\frac{1}{10}$ vol), the samples were stored over-
night at 4°C, and the TCA precipitable material was pelleted at
4-10°C by low speed centrifugation. To analyse for bound protein
content, the supernatant was carefully removed, and the preci-
pitate was resuspended in 35 μl sample buffer containing 1.5%
SDS, 15% glycerol, 0.001% brom phenol blue in the presence of
0.1 M unbuffered Tris and 1 M ß-mercaptoethanol. The sample was
incubated at 90°C for 5 min and applied to a slab gel (14 cm x
14 cm; thickness: 0.2 cm; 20% acrylamide, 0.5% bisacrylamide,
0.1% SDS in the presence of 0.4 M Tris-HCl, pH 8.8). The upper
3 cm of the slab gel was filled with spacer gel (6% acrylamide;
0.15% bisacrylamide; 0.1% SDS in the presence of 0.1 M Tris-HCl,
pH 6.8). 30 samples could be applied per slab gel. The electro-
phoresis buffer consisted of 0.05 M Tris-glycine, pH 8.8, and
0.1% SDS. Electrophoresis ran at 60 V for 1 h followed by 150 V
for 4 h. The gel was stained with 0.1% Coomassie Brilliant Blue
R250, 10% acetic acid and 50% methanol for 4 h at 60°C and de-
stained overnight at 60°C in 10% acetic acid and 10% methanol.
During destaining a polyurethane sponge was laid on the gel in
order to adsorbe the dye.

   If a protein bound to the 23S RNA under at least one con-
dition (see above), the binding assay was repeated with 7.5, 10
and 15 e.u. of the respective protein. A control assay was made
with 7.5 e.u. protein in the absence of RNA. The densities of
the resulting protein bands in the SDS gel were compared to that
of a band which was obtained from 5 e.u. of the respective pro-
tein in 35 μl sample buffer adjusted to pH 6.8, and to a 50S
subunit control (2 A_{260nm} units of 50S subunit in 35 μl sample
buffer, pH 6.8). Both the protein and the 50S sample were heat-
ed at 90°C for 5 min before application to the gel. The stoi-
chiometry of bound protein was estimated.

   In order to obtain a more precise measurement for the stoi-
chiometry of binding, some of the proteins were radioactively

labelled with [^{14}C]. The labelling was achieved by a reductive
methylation method which did not notably influence the activity
of the proteins (12). The specific activity of a protein was
determined assuming that one A_{230nm} unit is equivalent to 250 µg
protein. The RNA was incubated with increasing amounts of label-
led protein, and the incubation and the sucrose run were per-
formed as described above. The radioactivity in the fractions
was measured and the amount of bound protein calculated.

RESULTS

The binding assay was performed with 7.5 A_{260nm} units of
23S RNA (freed of 5S RNA), which was incubated with various
amounts of a highly purified protein under the conditions of
either the first or the second step of the two-step reconstitu-
tion procedure. The reconstitution mixture was layered onto a
sucrose gradient (5-20%), centrifuged, and the RNA and the bound
protein which co-migrated was precipitated with TCA. Non-bound
protein remained at the top of the tube. Controls without RNA
were run for each protein to verify that the fractions collected
for TCA precipitation did not contain non-bound protein.

The TCA precipitable material was applied to a slab gel in
order to estimate the amount of the bound protein. For details
see Materials and Methods.

In cases where binding occurred this technique allowed only
a rough estimate of the protein:RNA stoichiometry. We establish-
ed two categories: normal binding with an estimated stoichio-
metry of 0.7 to 1.3 (protein:RNA), and low binding with a range
from 0.1 to 0.6. When the assignment of a protein to one of these
two classes was ambiguous the respective protein was labelled
with [^{14}C] by the reductive methylation method. The RNA was in-
cubated with increasing amounts of the labelled protein. After
sucrose gradient centrifugation the radioactivity associated
with the RNA was determined and the stoichiometry of binding
calculated. Fig. 1A shows three examples, obtained with L13
(a nonbinding protein, as a control), L20 (which bound with a
stoichiometry of >2) and L24 (which shows a standard curve for
a binding protein). The resulting binding curves of three bind-
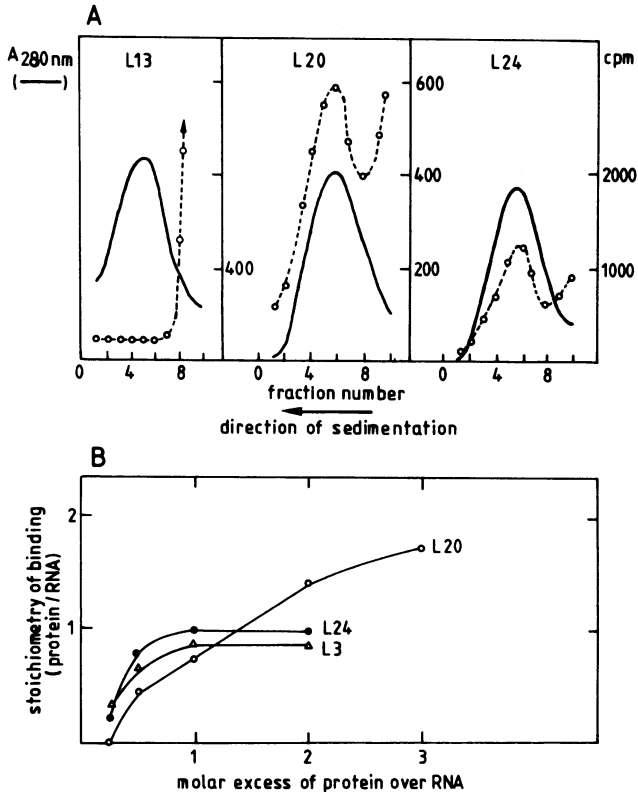
Figure 1: Determination of the stoichiometry of binding with [14C]-labelled proteins. A, sucrose gradient profiles of 23S RNA with either L13, L20 or L24. The molar ratio of RNA:protein during the incubation was 1:2, 1:2 and 1:0.5, respectively. Fractions of 15 drops were collected, mixed with 0.2 ml Soluene 350 and 5 ml Instagel (Packard) and counted. B, resulting curves of binding stoichiometry are given for the proteins L3, L20 and L24.

ing proteins (L3, L20 and L24) are presented in Fig. 1B.

The results are compiled in Table 1. 17 proteins bound to 23S RNA at 4 mM Mg^{2+} (first step condition), namely L1, L2, L3 L4, L7/L12, L9, L10, L11, L15, L16, L17, L18, L20, L22, L23, L24 and L29. Seven of these proteins bound stoichiometrically (L1, L2, L3, L4, L9, L23 and L24). L20 is an exception in that it could bind to the 23S RNA in more than one copy (see Fig. 1B).

Table 1: Binding of L-Proteins to RNA

Binding of protein / to	23S RNA (4 mM Mg^{2+})	23S RNA (20 mM Mg^{2+})	16S RNA (4 mM Mg^{2+})
L1	+	+	(±)
L2	+	(+)	−
L3	+	(+)	(±)
L4	+	(+)	−
L5	−	−	−
L6	−	−	−
L7/12	+	(+)	(±)
L9	+	(+)	−
L10	(+)	−	−
L11	(+)	(+)	−
L13	−	−	−
L14	−	−	−
L15	(+)	−	−
L16	(+)	−	−
L17	(+)	(+)	−
L18	(+)	−	−
L19	−	−	−
L20	+	+	−
L21	−	−	n.d.
L22	(+)	(+)	−
L23	+	+	−
L24	+	+	−
L25	−	−	−
L26	−	−	+
L27	−	−	−
L28	−	−	−
L29	(+)	(+)	−
L30	−	−	−
L31	−	−	−
L32	−	−	−
L33	−	−	−
L34	n.d.	n.d.	n.d.

Legend: +, stoichiometry of binding was 0.7 to 1.3 (protein:RNA). For L20 see text. (+), stoichiometry was 0.2 to 0.6.(±), stoichiometry was below 0.2; however, the protein amounts which migrated with the RNA, were significant. −, no binding to RNA. n.d., not determined. "4 mM Mg^{2+}" indicates the conditions of the first step, "20 mM Mg^{2+}" those of the second step of the two-step reconstitution procedure. For details see Materials and Methods.

At 20 mM Mg^{2+} nine out of the 17 proteins showed less affinity or even lost their binding ability (L2, L3, L4, L7/L12, L9, L10, L15, L16 and L18). Three proteins (L1, L3, L7/L12) showed low but significant binding to 16S RNA, and L26, as expected, strong interaction with 16S in contrast to 23S RNA. L26 is identical to S20 (ref. 13) which is known to be a binding protein of the small subunit.

Three observations are noteworthy: 1) In some cases the reductive methylation procedure strongly reduced the binding ability although the respective proteins are active in reconstitution. Examples are L7/L12 and L22. Clearly, the assembly of these proteins must be promoted by other proteins. 2) The binding affinity of some proteins varied considerably from preparation to preparation, whereas other proteins were not affected at all. The preparation sensitive proteins are L2, L7/L12, L10, L11, L15, L16 and L18. 3) L6 does not bind at all under either condition, L15 bound weakly only at 4 mM Mg^{2+}. However, when both proteins were added together, significant and equal amounts of both proteins bound to the 23S RNA (R. Röhl and K. H. Nierhaus, unpublished observation).

When 16S RNA was isolated from 30S subunit using acetic acid precipitation instead of the standard phenol treatment additional binding proteins are found (14). Therefore, we prepared (23S+5S) RNA with the acetic acid method (15). When this "acid" RNA was used in reconstitution assays less activation energy was required during the first step as compared to "phenol" RNA, whereas the activation energy for the second step was the same for both RNA preparations (15). We tested the binding of all ribosomal proteins to the "acid" RNA in presence of 4 mM Mg^{2+}. The result was that all 17 binding proteins bound also to the "acid" 23S RNA, whereas the other 50S proteins did not bind. Thus, no additional binding proteins were found with "acid" 23S RNA (G. Sieber, H. E. Roth and K. H. Nierhaus, unpublished observation).

DISCUSSION

The rRNA comprises two thirds of the total mass of the ribosome. Therefore, it can be expected that most if not all

ribosomal proteins carry an RNA binding site. If the protein re-
cognizes only a well-defined tertiary structure of the RNA as a
binding site, our assay system analysing an interaction between
one protein and the naked RNA is possibly not able to identify
the respective protein as a binding protein. It is probable that
the binding proteins are involved in the early stage of assembly.

The group of proteins referred to as "binding proteins" up
to now consists of eleven proteins (L1, L2, L3, L4, L6, L13, L16,
possibly L17, L20, L23 and L24). Our data demonstrate that two
(L6 and L13) of these proteins do not bind alone to naked 23S
RNA, whereas eight additional binding proteins could be identi-
fied (L7/L12, L9, L10, L11, L15, L18, L22 and L29).

What are the reasons for these discrepancies? Three points
seem to be of major importance: the incubation condition, the
preparation of the proteins, and the preparation of the RNA. The
importance of proper incubation conditions is evident from the
analysis of parameters influencing the total reconstitution of
active particles (11,16). Obviously, a milieu allowing the
assembly of active particles should be optimal for a study of
protein:RNA interactions. The importance of the method of pro-
tein preparation became clear when the introduction of a gentle
isolation technique led to the identification of two additional
binding proteins (L11 and L15; ref. 3). Furthermore, 16S RNA
isolated by acetic acid precipitation instead with the standard
phenol method bound additional 30S proteins (14).

In this paper we use for the first time 50S proteins which
are active in various reconstitution assays. Furthermore, RNA
and proteins have been incubated under conditions optimal for
the assembly of highly active particles (11). The use of 23S RNA
prepared by the acetic acid method did not reveal additional
binding proteins.

Two (L7/L12 and L9) out of the eight additional binding
proteins bound at 4 mM Mg^{2+} with a high affinity to 23S RNA,
and five of them showed binding affinities varying from one
preparation to another (L7/L12, L10, L11, L15 and L18).

Three proteins (except L26) were found to interact with
16S RNA (L1, L3 and L7/L12). Possibly, these proteins partici-
pate directly in the association of the subunits.

As mentioned above, the proteins identified as binding pro-
teins with the assay system used should be involved in the early
step of the assembly. A comparison with the proteins present on
the first reconstitution intermediate particles (ref. 9) reveals
in fact a good agreement: 13 out of the 17 binding proteins are
present on the $RI_{50}(1)$ particle which contains 18 proteins. The
binding protein L7/L12 could not be assigned unequivocally to
one of the assembly groups. In this context, the notion is of
particular interest that L6 and L15 although non- or low-binding
proteins, respectively, provoke a stoichiometric binding when
added simultaneously to the 23S RNA. One likely explanation of
this finding is that these proteins form a specific complex be-
fore binding to the 23S RNA, and that the complex has a higher
affinity for the RNA than either single protein. If the forma-
tion of such specific complexes were a common property of the
ribosomal proteins, these complexes would represent an important
feature of the cooperativity of ribosomal assembly. Specific
complexes of this type have been reported from 30S (17) and 50S
derived proteins (18, and G. Wystup, H. Teraoka, H. Schulze,
H. Hampl and K. H. Nierhaus, manuscript submitted).

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