NATURE OF THE RIBOSOMAL BINDING SITE FOR INITIATION FACTOR 3 (IF-3)*

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Summary: In vitro labelled IF-3 binds to both 16S and 23S rRNA but while one molecule of IF-3 binds to each 30S particle, binding to 50S particles is negligible. If proteins are removed by LiCl or CsCl treatment from either ribosomal subunit, however, binding specificity is lost and new "binding sites" appear on both ribosomal particles. Controlled RNase digestion of the 30S subunits does not cause the loss of any r-protein while controlled trypsin digestion results in the loss or degradation of several r-proteins; compared to the Phe-tRNA binding site, the binding site of IF-3 seems to be more sensitive to RNase than to trypsin digestion. Antibodies against single 30S r-proteins, which inhibit other ribosomal functions, do not prevent the binding of IF-3. RNA-binding dyes (acridine orange and pyronine) inhibit the binding of IF-3 to 30S ribosomal subunits. It is proposed that a segment of the 16S rRNA provides the binding site for IF-3 and that r-proteins confer specificity, restricting the number of available "binding sites", and stabilize the 30S-IF-3 interaction.

In vitro labelled IF-3 was shown to be bound to the 30S ribosomal subunits during the "30S initiation complex" formation and released upon formation of the "70S initiation complex" (1). Furthermore, several lines of evidence indicated that the interaction between 30S particles and IF-3 was specific: only one molecule of IF-3 was bound to each 30S subunits, and no stable binding was found between IF-3 and 70S monomers, 50S ribosomal subunits and with the 40S subunit of eukaryotic (pea seedlings) ribosomes. These results agreed with those reported by other laboratories (2,3). Since IF-3 interacts with RNA (4) and with proteins (5), the ribosomal binding site for IF-3 could be provided by either a specific segment of the 16S rRNA, some r-protein(s) of the 30S particle or by both (1).

The experiments described in the present paper were carried out in order to elucidate the nature of the interaction between 30S subunits and IF-3.

MATERIALS AND METHODS. E. coli (B or A19) ribosomes, ribosomal subunits and purified IF-3 were obtained as previously described (1,6). Labelling of IF-3 (1) by reductive alkylation (7) will be reported in detail elsewhere (8). CsCl (5.2M) 23S and 42S cores, and reconstituted ribosomes were prepared as described by Traub and Nomura (9) except that Mg^{2+} was 10mM. Before use, ribosomal subunits as well as CsCl and LiCl cores were "heat reactivated" by incubation at 50°C for 5 min. Binding of IF-3 to ribosomes was measured by sucrose gradient centrifugation (1).

Abbreviations: r-proteins=ribosomal proteins; IgG=immunoglobulin G (7S); Fab= monovalent antigen binding fragment (papain cleavage).

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RESULTS. Comparison of $^{14}CH_3$-IF-3 and "native" IF-3 binding sites. The labelled IF-3($^{14}CH_3$-IF-3)used in this study is an alkylated protein, which may differ from the "native"(not chemically modified)IF-3 in its interaction with 30S particles in spite of the preservation of its biological activity(1). However, since it was found that $^{14}CH_3$-IF-3 binds negligibly to the native(bearing initiation factors)30S subunits and that"native"IF-3 competes with it for the binding to 30S particles(data not shown), we assume that the ribosomal site to which $^{14}CH_3$-IF-3 is bound is equivalent to that of"native"IF-3.

Binding of IF-3 to 30S ribosomal subunits pretreated with hydrolytic enzymes. The 30S ribosomal subunits were subjected to controlled hydrolysis with either trypsin or RNases(A or T_1). The partially hydrolyzed 30S subunits"surviving" these treatments as particles sedimenting in the 20-30S range, were isolated from the more slowly sedimenting material and their IF-3, Phe-tRNA and fMet-tRNA binding capacities were assayed. As seen in Fig.1, preincubation of the 30S subunits with even the lowest amounts of RNases resulted in an extensive loss of both Phe-tRNA and IF-3 binding capacities. Increasing the amounts of nucleolytic enzymes beyond the concentration required to reach the plateaus of inhibition drastically reduced the yield of 20-30S particles, but did not substantially reduce the binding capacity of these"surviving"particles. In addition, RNases seemed to inhibit IF-3 and Phe-tRNA binding in a parallel way, since for each amount of enzyme used there was a close proportionality in the decrease of the two activities. Trypsin treatment, on the other hand, was found to affect Phe-tRNA more readily than IF-3 binding. Thus, 1 μg of trypsin(Fig. 1a)hydrolyzed the 30S subunits to a point where Phe-tRNA binding was virtually abolished(>80% inhibition), while little effect on IF-3 binding was observed (<20% inhibition). The binding of fMet-tRNA to ribosomes was inhibited by trypsin to a lesser extent than Phe-tRNA binding(Fig.1a)suggesting that the Phe-tRNA binding site itself rather than the mRNA binding site, was chiefly affected by trypsin treatment. Thus, the binding site for IF-3 on 30S ribosomes seemed to be more resistant to proteolytic digestion than the Phe-tRNA binding site.

IF-3 binding to CsCl and LiCl core particles. The removal from 30S subunits of several proteins(cf. Table 3)by either CsCl or LiCl treatment, produced subparticles(cores)without synthetic activity(Table 1)but did not result in a loss of IF-3 binding capacity(Table 1,2 and cf. Discussion). At low ratios of IF-3 to 30S particles no differences were found in the IF-3 binding activity of untreated 30S and CsCl 23S core particles(Fig.2). When the IF-3 concentration was increased, however, two plateaus were reached with 30S and 23S core

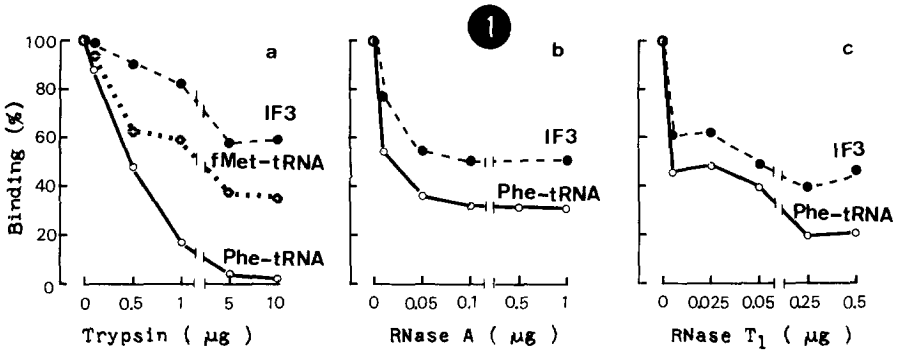

Figure 1. Effect of hydrolytic enzymes on the binding of Phe-tRNA, fMet-tRNA and IF-3. Each preicubation mixture, containing the indicated amounts of enzymes and 52(a), 77(b) and 25 A_{260} units of 30S ribosomes, was incubated at 37°C for 25 min(tryps.)or 10 min(RNases). The partially hydrolyzed particles sedimenting in the 20-30S range were isolated by sucrose gradient centrifugation and their poly(U)dependent binding of ^{14}C Phe-tRNA, poly(AUG)dependent binding of ^{3}H fMet-tRNA and $^{14}CH_3$-IF-3 binding(cf. Fig. 2)were determined. One hundred per cent binding corresponded to 16.5, 15.0 and 67.0 pmoles of Phe-tRNA, fMet-tRNA and IF-3 bound per A_{260} unit of 30S subunits.

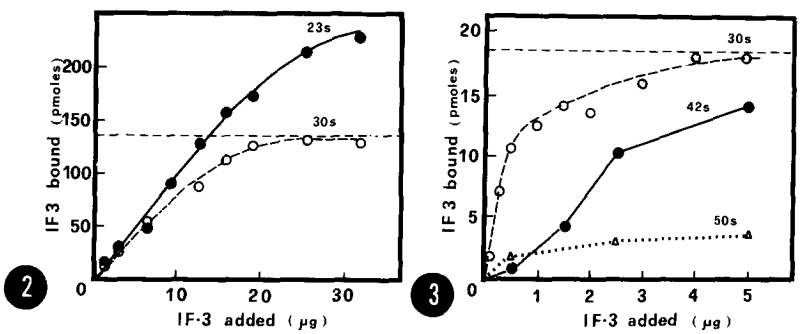

Figure 2. Binding of IF-3 to 30S particles and 23S CsCl cores. Each tube(0.4ml) contained in buffer(10mM TrisHCl, pH 7.8, 10mM $MgAc_2$, 100mM NH_4Cl, 5mM 2-mercaptoethanol)2.25 A_{260} units of either 30S or 23S particles and $^{14}CH_3$-IF-3(3,500 cpm/μg)as indicated. After 10 min at 37°C, the mixtures were loaded on 10-30% sucrose gradients(in the above buffer)and centrifuged at 48,000 rpm for 3 hr in the SW50.1 rotor. The A_{260} profile of each gradient was recorded continuously and the ribosome-bound radioactivity determined(1). The horizontal broken line indicates the approximate level of binding which would be expected if all the ribosomal particles had bound one molecule of $^{14}CH_3$-IF-3.

Figure 3. Binding of IF-3 to 30S and 50S subunits and 42S CsCl cores. Each tube for IF-3 binding(cf. Fig. 2)contained 0.27 A_{260} units of 30S or 0.54 A_{260} units of either 50S or 42S particles and the indicated amounts of $^{14}CH_3$-IF-3(21,000 cpm/μg). Centrifugation was at 48,000 rpm in the SW50.1 rotor for 3 hr(30S) 2 1/2 hr(42S)and 2 hr(50S).

particles respectively: the first plateau corresponds approximately to one molecule of IF-3 bound per 30S particle, the second to nearly double the amount of IF-3 bound per 23S core particle. Thus, rather than causing a decrese

in the IF-3 binding capacity, the removal of certain r-proteins results in the appearance of a new "binding site" for IF-3. This is probably due to the uncovering of certain 16S rRNA segments. In accordance with this finding, the formation of partially deproteinized particles (42S cores) by removal of some proteins from 50S subunits resulted also in a substantial binding of IF-3 (Fig. 3). The number of molecules of IF-3 bound per 42S core approaches one, whereas binding to intact 50S ribosomal subunits is negligible (Fig. 3).

Protein composition of ribonucleoprotein subparticles. The protein composition of the subparticles obtained by treatment of the 30S ribosomal subunits with different agents (enzymes and monovalent cations) was analyzed by two-dimension electrophoresis (10) and the results are presented in Table 3. As evident from the table, no clear relation can be found between the presence (or absence) of any r-protein(s) and the binding of IF-3. Although several r-proteins are either absent or greatly reduced in CsCl and LiCl cores, and after treatment with low concentration of trypsin, IF-3 binding was not diminished. The treatment of the 30S particles with nucleolytic enzymes, on the other hand, did not produce any major change in the protein composition of the particles but substantially reduced their IF-3 binding capacity.

Effect of antibodies against 30S r-proteins on IF-3 binding. A way of determining whether a particular protein or a group of r-proteins is involved in a given ribosomal function is to look for a specific decrease of this function after incubation of the ribosomes with IgGs or their monovalent Fab fragments prepared from antisera against single r-proteins (11-13).

Antibodies (Fab fragments) prepared against all single 30S ribosomal proteins were tested for a specific inhibition of IF-3 binding. In spite of the fact that nearly all the Fab tested almost completely abolished poly(U) dependent polyphenylalanine synthesis (14), and that several of them were found to affect poly(U) dependent Phe-tRNA binding, none of them clearly inhibited IF-3 binding. Minor (6-20%) and not additive inhibitions were obtained with Fab against proteins S4, S7, S12, S13, S14 and S19; Fab against protein S18 gave inconsistent results showing either inhibition or stimulation of IF-3 binding.

Effect of nucleic acid binding dyes on IF-3 binding. In Fig. 4 the effect of increasing amounts of acridine orange (at 10mM Mg^{2+}) on the ribosomal binding of IF-3 is shown. It is evident that as the concentration of the dye was increased, IF-3 binding to 30S particles was substantially reduced, whereas little or no effect on the binding of a small amount of IF-3 to 50S particles was observed. In these experiments, dye was added to the 30S particles before the addition of IF-3. The inhibition of IF-3 binding was less pronounced, how-

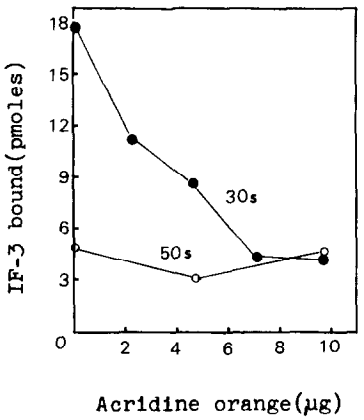

Figure 4. Effect of acridine orange on the binding of IF-3. Each tube, containing 0.3 A_{260} units of 30S or 0.6 A_{260} units of 50S subunits, was incubated at 37°C for 5 min with the indicated amounts of acridine orange prior to the addition of 2 μg of $^{14}CH_3$-IF-3(3,500 cpm/μg).

ever, if acridine orange was added to the ribosomes after IF-3. Pyronine G, another RNA-binding dye, was similarly found to inhibit IF-3 binding(not shown).

IF-3 binding to rRNA. The binding of IF-3 to 16S and 23S rRNA was studied by sucrose gradient centrifugation and by Millipore filtration. Both methods clearly showed that IF-3 binds to both 16S and 23S rRNA, but proved inadequate to determine the stoichiometry and specificity of the binding. At least 6-8 molecules of IF-3 were necessary to retain one molecule of ^{32}P 16S rRNA(twice as many for 23S rRNA)on a Millipore filter. The calculation of the stoichiometry of the IF-3-rRNA complex by sucrose gradient centrifugation, on the other hand, was impossible because the complexes were partially dissociated during centrifugation and a large amount of radioactivity trailed after the A_{260} peaks.

DISCUSSION. In this study we have often used the nonenzymatic, poly(U)dependent binding of Phe-tRNA to 30S subunits as a reference point to study IF-3 binding. This was made with the assumption that the Phe-tRNA binding site is mainly contributed by r-proteins(15,16)although an additional role of the rRNA cannot be ruled out(17,18). Of the 21 r-proteins identified in purified 30S particles (10), only some(S4,S5,S7,S8,S9,S15,S16,S17,S20)are present in amounts approximating one copy per particle(16,19). Since one molecule of IF-3 binds to each 30S particle(1-3), only one of the above proteins could be responsible for the binding of IF-3 if we assume that IF-3 binds to a single r-protein. Alternatively, a heterogeneous group of r-proteins could provide the binding site for IF-3. In both cases, an inhibition of IF-3 binding upon incubation of the ribosomes with antibodies against single r-proteins should be expected(in the 30S particle all the r-proteins are accessible to their respective antibodies(20)).

Table 1. Binding of IF-3 and synthesis of polyphenylalanine with 23S CsCl cores, control and reconstituted 30S particles.

Particle	^{14}C polyphe formed (cpm/A_{260} unit of particles)	$^{14}CH_3$-IF-3 bound (cpm/A_{260} unit of particles)
30S control	61,015	1,858
23S CsCl core	670	1,842
30S reconstituted	48,908	1,098

Polyphenylalanine synthesis was measured (9) in the presence of a slight molar excess of 50S ribosomal subunits. Binding of IF-3 was as described under Fig. 2 but each incubation mixture contained 2.25 A_{260} units of ribosomal particles and 6 µg of $^{14}CH_3$-IF-3 (3,500 cpm/µg).

Table 2. Binding of IF-3 to LiCl core particles.

$^{14}CH_3$-IF-3 bound (cpm/A_{260} unit)	Core particles obtained with LiCl					
	0.0 M	0.4 M	0.6 M	0.8 M	1.0 M	1.5 M
	1,838	1,727	2,364	1,488	1,940	2,239

LiCl cores were prepared dissolving 30S subunits with buffer A(10mM TrisHCl, pH 7.8; 10mM $MgAc_2$; 60mM NH_4Cl; 6mM 2-mercaptoethanol) containing the above concentrations of LiCl. After 5 hr incubation at 0-2°C, the particles were collected by centrifugation for 6 hr at 50,000 rpm in the Spinco 50ti rotor and resuspended in buffer A. IF-3 binding was measured as indicated under Table 1.

Table 3. Protein composition of the ribosomal subparticles obtained from 30S ribosomal subunits with various treatments.

Treatments:	Ribosomal proteins																				
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11★	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21
CsCl (5.2 M)	o	o	o	±	o	+	+	+	±	o	±	±	+	o	±	±	+	+	o	±	±
LiCl (1.5 M)	o	±	±	+	±	+	+	±	+	o	o	o	o	o	±	+	+	±	o	+	±
Trypsin (0.5µg)	o	±	±	+	+	±	+	+	±	±	o	+	+	+	+	±	±	+	±	+	±
Trypsin (10 µg)	o	o	±	+	±	o	±	+	±	±	o	±	±	+	±	±	±	±	o	+	o
RNase A (1 µg)	±	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RNase T_1(0.5µg)	±	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Two-dimensional polyacrylamide gel electrophoresis were as described (10). +, present in normal amounts; ±, present in reduced amounts; o, present in trace amounts or absent. ★The presence of S11 is difficult to determine under the conditions used and can only be surmised.

No antibody against single 30S r-proteins,however,was found to significantly prevent the binding of IF-3 to 30S subunits. In addition,no relation between the loss from 30S particles of any specific r-protein(s) and the binding of IF-3 was established,and the partial digestion of the 30S particles with either proteolytic or nucleolytic enzymes seemed to indicate a greater sensitivity of the IF-3 binding site to the action of nucleolytic enzymes. Furthermore,the binding of IF-3 was inhibited by dyes known to interact with nucleic acids but not with proteins(21,22). IF-3 binds to both 16S and 23S rRNA,but a specific binding occurs only with the 30S and not with the 50S ribosomal subunits. This specificity,however,is lost when some r-proteins are removed from either subunit and new"binding sites"appear on both 23S and 42S cores.

Taken together,these data seem to be compatible with the premise that the binding site for IF-3 on the 30S ribosomal subunit is mainly provided by a segment of the 16S rRNA. The function of the r-proteins could be,as the experiments with CsCl cores indicate,to confer specificity to the binding of IF-3 by restricting the number of possible"binding sites",and,since IF-3 binds more strongly to 30S particles than to isolated 16S rRNA,to increase the stability of the binding providing auxiliary binding forces and keeping the rRNAbinding segment in a suitable conformation.

This conclusion is consistent with the knowledge that IF-3 is a basic protein that would be expected to interact with nucleic acids through electrostatic bonds. That this is probably the case is supported by our unpublished observation that the binding to 30S particles is inhibited by high ionic strength(80% inhibition at 0.4M NH_4Cl),whereas moderate changes in temperature or Mg^{2+}concentration have little effect(1-3).

Finally,it should be noted that some data of this paper:a)the variability of IF-3 binding to LiCl cores(cf.Table 2),b)the seemingly paradoxical finding that"reconstituted"30S particles have a lower affinity for IF-3 than control 30S and 23S core particles(cf.Table 1)and perhaps c)the puzzling effect of Fab against protein S18(cf.text)seem to indicate that the configuration of the ribosomal particles plays an important role in the binding of IF-3.

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