THE RELEASE OF PROTEINS AND 5S RNA DURING THE UNFOLDING OF *ESCHERICHIA COLI* RIBOSOMES

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1. Introduction

When ribosomal subunits from *Escherichia coli* are dialysed extensively against either low salt concentrations (1 mM EDTA, 5 mM Tris–HCl, pH 7.6), or against high salt (1 M NH_4Cl, 0.1 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.6), a more open ribosomal structure is formed that sediments more slowly than the untreated ribosomal subunits [1–6]. This structural change has been termed 'unfolding'.

It has been demonstrated that almost all of the 5S RNA is released from the 50S subunits during unfolding in the presence of both EDTA [7] and 1 M NH_4Cl [3]. Although there is no report, to date, of any proteins being co-released during EDTA-unfolding [2], there is evidence of 4–7 unidentified proteins being released by the high-salt treatment [3].

Since it is known that a group of proteins are bound to 5S RNA in the 50S subunit [8–11], and facilitate complex formation of 5S RNA with 23S RNA [8,12], we attempted to identify which proteins, if any, are released during unfolding, in order to establish whether they correspond to the proteins that bind to 5S RNA.

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It was found that a few proteins were released with the 5S RNA during both unfolding procedures, only one of which, L25, binds strongly to 5S RNA. The other 5S RNA-binding proteins remained attached to the 50S subunit. During high salt unfolding, protein L30 that assembles weakly with 5S RNA [10] was also detached. Functional studies, reviewed in [13], suggest that the other proteins that were wholly or partially removed may lie within a functionally important region in the vicinity of 5S RNA within the 50S subunit.

2. Materials and methods

2.1. *Preparation of unfolded 70S ribosomes and 50S subunits*

70S ribosomes and 50S subunits were prepared as described earlier [8].

2.1.1. EDTA treatment

The 50S subunits (or ribosomes) were dissolved in 10 mM Tris–HCl, pH 7.6 and 1 to 5 mM EDTA at 10–60 mg/ml and treated as follows. 1) The ribosome sample (2–6 ml) was dialysed (16 hr) against this buffer, and passed over an A 1.5 agarose (Biorad, California) column (2 × 180 cm). The ionic strength of the unfolded ribosomes, that were eluted in the excluded volume, was adjusted to 10 mM $MgCl_2$ and

0.1 M KCl and the ribosomes were precipitated with 0.7 vol ethanol (1 hr at 4°C) and centrifuged. 2) The ribosome sample (2.5 ml at 50 mg/ml) was applied to a G100 Sephadex column (3 X 120 cm) that had been equilibrated for two days with the EDTA-buffer. The unfolded ribosomes were eluted in the excluded volume and centrifuged. 3) The ribosome solution (2 ml at 60 mg/ml) was dialysed (4 hr) against the EDTA-buffer, 0.3 ml was centrifuged for 16 hr at 20 000 rev/min in each tube of an SW 27 swinging-bucket rotor containing a 5–30% w/w sucrose density gradient in the EDTA-buffer. The unfolded ribosomes were separated from the gradient, dialysed (8 hr) against 30 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ and centrifuged overnight.

Proteins were extracted in 67% acetic acid [14] and identified electrophoretically [15]. The proteins extracted from the untreated ribosome sample were also electrophoresed for comparison.

2.1.2. 1 M NH_4Cl treatment

The 50S subunits were dissolved in 1 M NH_4Cl, 10 mM Tris–HCl, pH 7.4, 0.1 mM magnesium acetate. The solution (3 ml at 10 mg/ml) was dialysed against this buffer for 24 hr and passed over an A 1.5 agarose column (2 X 60 cm) which had previously been equilibrated (18 hr) with this buffer. The unfolded ribosomes eluted in the excluded volume were dialysed against 0.1 M NH_4Cl, 0.1 mM magnesium acetate (8 hr) and then precipitated (24 hr) with 0.7 vol absolute ethanol. The proteins were extracted and identified as described above.

2.2. *Isolation of proteins released during unfolding*

Ribosomes that had been unfolded by either EDTA or high salt, were applied to A 1.5 agarose columns as described above. The included volume, between the unfolded ribosome peak and a calibration 2-mercaptoethanol peak was pooled and dialysed against 2% acetic acid for 16 hr (EDTA-unfolded sample) and 36 hr (high salt-unfolded sample). The solutions were then lyophilised and the proteins identified electrophoretically [15]. All proteins except S1 were detectable by this method.

2.3. *Binding assay for 5S RNA-23S RNA and 5S RNA-unfolded ribosome complex formation*

Binding assays were performed by sucrose density gradient centrifugation and by filtration through nitrocellulose filters [8]. The reaction mixture contained 1 A_{260} unit of unfolded particles or 23S RNA, about 0.1 molar equivalent of [^{32}P]-5S RNA and 1 molar equivalent of the proteins to be tested in 0.1 ml buffer (30 mM Tris–HCl, pH 7.6, 20 mM $MgCl_2$, 300 mM KCl and 6 mM 2-mercaptoethanol). The samples were incubated at 35°C for 15 min. One set of duplicate reaction mixtures was chilled, diluted with 3 ml of the assay buffer, and filtered through 0.45 μm Millipore filters with three washes of 3 ml each. The second set of duplicate reaction mixtures was sedimented through 5–20% sucrose in 10 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 30 mM KCl and 6 mM 2-mercaptoethanol in a Spinco SW-50 L rotor at 49 000 rev/min for 2.5 hr at 1°C. The tube content was scanned at 254 nm and fractionated into 0.25 ml fractions and the radioactivity measured [8].

3. Results

3.1. *Protein content of unfolded ribosomes and the released proteins*

3.1.1. EDTA treatment

70S ribosomes and 50S subunits that had been unfolded by EDTA treatment were separated from the released proteins by three methods (see Materials and methods) which gave identical results. Each of the 50S subunit proteins was present in the unfolded ribosome except L25. A few other proteins had been partially removed (table 1). The identities and quantities of the proteins released during unfolding were completely compatible with these results (table 1). The same results were obtained with 50S subunits and 70S ribosomes. Moreover, no 30S subunit proteins were released from the latter.

3.1.2. 1 M NH_4Cl treatment

The 50S subunits (or 70S ribosomes) that had been unfolded in high salt buffer were separated from dissociated proteins. Four proteins, namely L6, L25, L28 and L33 were completely absent from the unfolded ribosomes. Other proteins were partially removed (table 1). The identities and quantities of the released proteins were in agreement with these results, except that trace amounts of a few additional proteins were detected (table 1).

Table 1
The identities of the proteins that were partially or completely released during the unfolding of 50S subunits

Protein	EDTA-unfolding		1 M NH_4Cl-unfolding	
	Unfolded subunits	released proteins	unfolded subunits	released proteins
L2	++	–	+(+)	(+)
L4	+	+	++	–
L5	++	–	++	(+)
L6	++	–	–	++
L7	+	+	+	+
L8/L9	+	+	+	+
L12	+	+	+	+
L14	++	–	+	–
L18	++	–	++	(+)
L19	++	–	++	(+)
L21	++	–	++	(+)
L25	–	++	–	++
L26	++	–	+	+
L27	++	–	+	+
L28	++	–	–	+
L30	++	–	(+)	++
L33	++	–	–	++

The amount of each protein present was estimated from the intensity of its spot in a 2-D polyacrylamide gel relative to a control gel of total 50S subunit protein. The notation system is as follows: ++ normal intensity, + about half the normal intensity, (+) very weak spot and – no spot. The amount of L4 present in the EDTA-unfolded subunits was variable but always less than in the control.

The results were the same whether 50S subunits or 70S ribosomes were taken. However, in contrast to the EDTA-unfolding experiments S3, S5 and S10 were completely displaced from 70S ribosomes together with substantial amounts of S2 and S9/S11.

3.2. *Binding of 5S RNA to high salt-unfolded ribosomes*

It was previously shown that 5S RNA can be linked to 23S RNA through a group of proteins one of which, L18, is essential [8]. The other proteins are L5 and L25 [8; J. Feunteun, unpublished work]. Protein L6 attaches to 23S RNA (16) and may stabilize this complex [8]. Only protein L25 was co-released with the 5S RNA. During the high salt unfolding L6 was also released. Neither L5 nor L18 were detached; therefore they must form part of the binding site of the 5S RNA–L25 complex within the 50S subunit. Some binding experiments were performed in order to establish whether the 5S RNA could reassemble with the unfolded ribosome. Sucrose density gradient and nitrocellulose filtration experiments showed, in agreement with the earlier binding experiments [8], that the proteins dissociated in 1 M NH_4Cl did not induce 5S RNA–23S RNA complex formation but that they could effect the reassembly of 5S RNA to the unfolded 50S subunit.

4. Discussion

It was shown earlier that 5S RNA is displaced from the 50S subunit during both EDTA and high salt unfolding; the co-released proteins have now been identified. Only one of the proteins that binds strongly to 5S RNA [8–11] was released during both procedures, namely L25. L30, which assembles weakly to 5S RNA [10], was also released during high salt unfolding. Since the two other proteins that bind strongly to 5S RNA, namely L5 and L18, remain in the unfolded subunit it can be concluded that the mechanism of both unfolding processes involves the disruption of these two protein–RNA interactions.

A number of proteins that either bind directly to 5S RNA [8,9], or assemble with 5S RNA in the presence of other proteins [10] have been implicated, at least indirectly, in an important functional region of the 50S subunit [17]. Other proteins indirectly related to the 5S RNA–protein complex by both structural and functional studies [8,18,19] have also been implicated in the amino acyl-tRNA acceptor and donor sites on the 50S subunit [reviewed in 13]. It is noteworthy that most of the proteins released on unfolding, namely L4, L6, L7, L8/L9, L12, L25, L27, L30 and L33, fall into one of these two categories. It seems likely, therefore, that part of this functional region on the 50S subunit is destabilised and becomes detached from the subunit during unfolding.

In conclusion, it is noteworthy that the co-release of functionally important proteins with 5S RNA during unfolding may explain the failure of attempts to reconstitute unfolded ribosomal subunits into functionally active ribosomes [7,20,21].

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