The presence of RNase II in high salt washed *E. coli* ribosomes: effect on circular dichroism of ribosomal complexes

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Received 13 January 1977

ABSTRACT

Traces of the exonuclease RNase II are retained by *E. coli* ribosomes purified by conventional high salt washing. The residual activity can give rise to serious artifacts, especially in physical studies.

INTRODUCTION

For some time it has been recognized that crude ribosome preparations contain nuclease activity.¹ In order to remove this activity, treatment of crude bacterial ribosomes with high concentrations of salts such as ammonium chloride was adopted.²⁻⁴ This treatment was found to remove other contaminating proteins as well as initiation factors, and is widely used to provide a reasonably uniform and factor-free preparation.² It has now come to be recognized that high salt washing, which is so conveniently used for purification of ribosomes, can unfortunately also cause depletion of intrinsic ribosomal proteins.^{2,5} There seems to be correspondingly little concern about how efficiently nucleases are removed by high salt washing. In the course of circular dichroism measurements of template polynucleotide and tRNA binding to *E. coli* ribosomes, we found that spectral changes which we first assigned to conformational changes in the ribosome were due instead to degradation of polynucleotide by traces of the exonuclease RNase II remaining in the high salt washed preparations. Moreover, we determined that nuclease activity sufficient to produce such artifacts is generally present in high salt washed *E. coli* ribosomes prepared by others.

Contamination of ribosomes by the endonuclease RNase I can be easily avoided by use of *E. coli* strains deficient in RNase I, as is commonly done. But these strains still produce other nucleases including RNase II. The degradation of template polynucleotide in crude amino acid incorporation systems, described some time ago by Nirenberg,¹ is traceable to contamination by RNase II.⁶ In their characterizations of RNase II activity, Spahr⁷ and Singer⁶ assessed its distribution during cytosol fractionation and found that

a sizable proportion of the total activity remains with the ribosomes even after several cycles of dissolution and pelleting at low to moderate salt concentration. The original descriptions of techniques for high salt washing^{3,4} do not really assure complete nuclease removal by the usual procedure of two or three cycles of dissolution and pelleting; and it appears⁸ that many such cycles of high salt washing may be required. There is also independent evidence that a limited amount of RNase II tightly bound to the ribosome is protected from inactivation.

RESULTS AND DISCUSSION

We wished to examine conformational changes that might occur in the ribosome during the various stages of protein synthesis. We chose ultraviolet circular dichroism (CD) as a probe for gross conformational alterations, and selected the nonenzymatic binding of poly(U) and tRNA^{phe} to the E. coli 70S ribosome for our initial studies. We purified crude ribosomes prepared from E. coli MRE-600 (an RNase I deficient strain) by two cycles of dissolution and pelleting in 2M NH₄Cl, being unaware that appreciable RNase II activity would remain. Addition of poly(U) to these ribosomes produced small but significant CD differences (Fig. 1a). Addition of tRNA^{phe} to the ribosome-

Fig. 1: CD difference spectra for binding mixtures of high salt washed E. coli ribosomes, poly(U) and tRNA^{phe} at 25°, pH 7.0 and 20 mM Mg²⁺. Bars, observed difference spectra, showing error limits. Curves, spectra calculated from poly(U) degradation. (a) difference between ellipticity of ribosome-poly(U) mixture and sum of ribosome and poly(U) ellipticities, (b) difference between ellipticity of ribosome-poly(U)-tRNA^{phe} mixture and sum of ribosome-poly(U) mixture and tRNA^{phe} ellipticities. Curve (a) represents total degradation of poly(U); curve (b), 30% retention.

poly(U) mixture led to differences of another form (Fig. 1b); acylation of the tRNA^{phe} with phenylalanine did not significantly alter the result. Binding of tRNA^{phe} or phe-tRNA^{phe} in the absence of poly(U) did not give significant CD differences. We tentatively concluded that the observed CD changes represented conformational changes in the ribosome, produced on binding of poly(U) and on binding of tRNA^{phe} to the ribosome-poly(U) complex.

That these CD changes are, instead, an artifact occurred to us only when we looked at the CD effect of binding poly(C). The result with poly(C) was a very large negative CD difference centered around 275 nm, indicating the loss of secondary structure of the poly(C). Poly(U) binds much better to ribosomes than other polyribonucleotides because of its relative lack of secondary structure.⁹ Since weak binding of poly(C) would not be expected to lead to such a large loss of secondary structure, we considered degradation of the poly(C), and of the poly(U) as well, as the possible explanation of the CD changes. To test this hypothesis, poly(U) that had been exposed to the high salt washed ribosomes for 1 h under the conditions of the CD experiments was fractionated by gel filtration (Fig. 2). The poly(U) was nearly completely converted to monomer or very short chain fragments; the presence of tRNA^{phe}, however, limited the conversion to about 70%. These results indicated that the CD effects seen with the ribosomal binding mixtures could be explained by degradation of the template polynucleotide. In fact, CD difference spectra approximating those generated by the ribosome systems can be

Fig. 2: Fractionation of [³H] poly(U) degradation products from high salt washed ribosome binding mixtures, by gel filtration on Sephadex G-25. ●, ribosome-poly(U) mixture, at zero time; O, ribosome-poly(U) mixture, after 1 h; ■, ribosome-poly(U)-tRNA^{phe} mixture, after 1 h at 25°. The (Up)₇U elution position is given as a reference.

calculated from the extent of poly(U) degradation observed for the ribosomal binding mixtures and the changes in the CD spectrum of poly(U) produced by degrading it with pancreatic RNase, as shown in Fig. 1. Thus the CD differences generated by the addition of poly(U) to high salt washed ribosomes are consistent with near total degradation of the poly(U) while the differences generated by addition of tRNA^{phe} to the ribosome-poly(U) mixture are explained by protection of some of the poly(U) in a tighter ribosome-poly(U)-tRNA^{phe} complex (see also ref. 10).

To determine whether the polynucleotide degrading activity was a peculiarity of our own ribosome preparations, or whether it is generally present in high salt washed preparations, we examined such preparations obtained from several other investigators, from M. Cashel, from E. Hamel, and from H. Weissbach and N. Brot. All of these preparations possessed degradative activity against poly(U) when measured under our binding conditions, so that the poly(U) was degraded to small products and CD differences of the type of Fig. 1a were generated.

In order to repeat the CD measurements under conditions where the poly(U) would not be degraded, we attempted to separate the degradative activity from high salt washed ribosomes by band sedimentation through a sucrose gradient (Fig. 3). This procedure left the poly(U) degrading

Fig. 3: Redistribution of poly(U) degrading activity from salt washed ribosomes by sedimentation of the ribosomes through a sucrose gradient. The distribution of 70S ribosomes as well as some 50S and 30S subunits is measured by the A_{260} of the fractions; degradative activity is measured by the decrease in acid precipitability of poly(U) following incubation with the fractions.

Fig. 4: CD difference spectra for ribosome binding mixtures at 25°, pH 7.0 and 20 mM Mg²⁺, using high salt washed *E. coli* ribosomes freed of nuclease activity by band sedimentation through a sucrose gradient. Display as in Fig. 1. (a) ribosome-poly(U) system, (b) ribosome-poly(U)-tRNA^{phe} system.

Fig. 5: Fractionation of [³H] poly(U) degradation products from high salt washed ribosome-poly(U) mixture, by gel filtration on Bio-Gel agarose A-1.5m. ○, at zero time; ●, after 3 min; ■, after 8 min; □, after 16 min at 25°.

activity near the top of the gradient, as Spahr⁷ and Singer¹¹ had found for RNase II separated in this way from crude ribosomes. Binding of poly(U) or poly(U) plus tRNA^{phe} to high salt washed ribosomes further purified by sedimentation through a sucrose gradient gave no significant CD differences, as shown in Fig. 4. Thus, there is no evidence that binding of these substances to high salt washed ribosomes produces CD differences other than those due to the degradation of poly(U).

We further characterized that degradative activity as RNase II by following the disappearance of poly(U) and the generation of its degradation products. Gel filtration using a matrix of high exclusion limit showed (Fig. 5) that the degradation of poly(U) was an exonucleolytic process. The degradation gives small product throughout its course, without accumulation of long chain intermediates, and goes to near complete conversion to small product before exhaustion of the poly(U). The end product from poly(U) was established as 5'UMP by thin layer chromatography (Table I). The mode of degradation and the 5'-mononucleotide end product are entirely consistent with the processive attack of polyribonucleotides from the 3' terminus which is characteristic (6,7,12) of RNase II.

TABLE I.		Thin Layer Chromatographic Identification of Poly(U) Monomeric Product			
		cpm from [³ H] poly(U)			
Area cut Location	origin 0-2½cm	5'UMP 2½-7cm	3'UMP 7-11½cm	(Blank) 11½-15¾cm	(Blank) 15¾-20cm
poly(U) + ribosome	679	4002	468	47	67
poly(U) alone	6924	122	84	52	42

CONCLUSION

These results indicate that conventional high salt washed *E. coli* ribosomes may be expected to retain traces of RNase II. The presence of traces of RNase II in a ribosome preparation can give rise to serious artifacts, especially if an effect is being detected by optical means and discrimination of small changes is required.

Despite the large amount of literature on ribosome preparation, some potentially undesirable procedures may be in fairly common use. While dissolution and pelleting from high salt media is conveniently used in

ribosome purification, there are at least two problems with the procedure.

(1) The ribosome is exposed to a salt concentration at which ribosomal components may dissociate. (2) Pelletting through the solution allows continued contact between ribosome and contaminant particles. If the removal of ribosomal proteins is to be prevented, high salt concentrations must be avoided (although RNase II could be removed efficiently by chromatography, rather than pelletting, under high salt conditions^{4,12,14}). The method of choice for exhaustively removing RNase II but still avoiding removal of ribosomal proteins appears to be band sedimentation through a sucrose gradient at low salt concentration; this procedure has been explored by Singer¹¹, who has used it successfully to purify ribosomes for polynucleotide binding experiments.¹⁰

Consideration of these problems leads also to the perhaps obvious conclusion that no procedure for purifying a complex entity such as the ribosome may be wholly satisfactory for all purposes. Therefore the preparation method has to be optimized for each particular use for which ribosomes are prepared -- thus, for use in the spectroscopic determination of conformational changes involving template RNA binding, ribosomes must be freed of enzymes attacking the RNA -- and, if need be, at the expense of some properties of the ribosome.

EXPERIMENTAL

Materials

High salt washed ribosomes were prepared from late log phase *E. coli* MRE-600 (General Biochemical). Cell suspensions in buffer A were ground with glass microbeads in a blender¹⁵ and then subjected to the procedure of Patterson and Gillespie¹⁶, which includes preliminary treatment with pancreatic DNase I at 0°, incubation of the 30,000 x g supernatant with ATP and phosphoenolpyruvate, and the use of HEPES buffers throughout. Ribosomes were washed¹⁶ with ammonium chloride, once with 60 mM, then twice with 2M, and then once again with 60 mM, in Buffer B; and stored at -20° in the final buffer plus 50% glycerol. High salt washed *E. coli* ribosomes obtained from other investigators originated from mid or late log phase cells.

tRNA^{phe} (yeast) was from Boehringer-Mannheim; it was acylated with phenylalanine using yeast phenylalanine-tRNA synthetase (purified through the DEAE-Sephadex stage of ref. 17), and the phenol extracted product precipitated with ethanol and passed through Sephadex G-25. Poly(U), [³H] poly(U) and poly(C) were from Miles Laboratories. (Up)₇U was from Boehringer Mannheim. 3'UMP, 5'UMP, and pancreatic DNase I and RNase A were from Sigma. Sucrose for density gradients was RNase-free, density gradient

quality from Schwarz-Mann. Buffer A is 10 mM magnesium acetate, 60 mM ammonium chloride, 3 mM 2-mercaptoethanol, 30 mM HEPES, pH 7.0. Buffer B is 10 mM magnesium acetate, 3 mM glutathione, 30 mM HEPES, pH 7.0. Buffer C is 20 mM magnesium acetate, 110 mM ammonium chloride, 5 mM 2-mercaptoethanol, 50 ml HEPES, pH 7.0.

Circular Dichroism

Ultraviolet CD spectra were measured with a Cary 61 instrument. Difference spectra of ribosome binding mixtures were recorded directly. Components in buffer C were mixed at 0°, warmed to ambient temperature of approximately 25° for 20 min, and the difference spectrum recorded over a 40 min period from 300 to 230 nm, on the 10 mdeg scale, at a time constant of 30 sec. For high salt washed ribosomes, three 0.5 mm cuvettes were employed; the binding component concentrations were 400 nM 70S ribosomes, 150 μ M (residue) poly(U), 3 μ M tRNA^{phe} (when phe-tRNA^{phe} was included it was present as 20% of the total tRNA^{phe}; see ref. 18,19). For sucrose gradient purified ribosomes, three 1.0 mm cuvettes were employed with binding component concentrations half those used with the high salt washed ribosomes, in order to accommodate the ribosome concentrations obtainable from the gradient fractions. In the figures, the mean ellipticity difference is given at 5 nm intervals by vertical bars of length twice the mean deviation. The data in Fig. 1 represent 7 determinations for the ribosome-poly(U) system and 12 determinations for the ribosome-poly(U)-tRNA^{phe} system; in Fig. 3, 4 determinations for each system. $\Delta(\theta)$ of 1×10^6 on the left hand (ribosome) scale is equivalent to a measured $\Delta \theta$ of 0.2 mdeg.

Independent CD difference spectra for poly(U) degradation were calculated from CD spectra of a 75 μ M (residue) poly(U) solution measured separately in buffer C, at 10 mm light path, before and after addition of 0.2 μ g pancreatic RNase A per ml poly(U) and incubation for 2h at 37°.

Sucrose gradient sedimentation

For CD studies in the absence of nuclease activity, 100 A₂₆₀ units high salt washed ribosomes were sedimented into each 30 ml 5-20% linear sucrose gradient in buffer C, at 75,000 x g for 6h at 4°. The peak fractions from the 70S region were pooled and used without reconcentration or change of solvent; the preparation was stored at 0° up to 2 days as required. Lack of degradation of poly(U) by this preparation under the conditions of the CD determinations was checked by gel filtration on Sephadex G-25. For assessing the distribution of poly(U) degrading activity in a sucrose gradient (Fig. 3), 50 A₂₆₀ units of the salt washed ribosomes were sedimented as above. Degradative activity was measured by the decrease in [³H] poly(U) precipitated by

trichloroacetic acid-uranyl acetate²⁰ after incubation of 500 pmole (residue) poly(U) for 1 h at 25° with each 1 ml gradient fraction, the precipitate collected, washed, and counted on a nitrocellulose filter.

Chromatography of poly(U) degradation products

Gel filtration was carried out on a 1 x 25 cm column of Sephadex G-25 and on a 2 x 25 cm column of Bio-Gel agarose A-1.5m, in buffer C. Reaction mixtures, 0.2 and 0.5 ml respectively, were of the same composition as in the CD determinations, with the addition of 2 μ M (residue) [³H] poly(U) (62 Ci/residue) to the nonradioactive poly(U). 1.0 ml fractions were collected and counted directly in Aquasol scintillant. The [³H] poly(U) used for the agarose chromatography was first refracted on the column to provide a sharper initial distribution.

Thin layer chromatography was carried out on 100 μ M thick cellulose layers pretreated by spraying with saturated aqueous sodium borate and drying. Reaction mixtures, approx. 10 μ l, of the same composition as in the CD determinations, using [³H] poly(U) (62 Ci/residue, undiluted), were incubated for 1 h at 25°. 3'UMP and 5'UMP were separated one-dimensionally by ascending development with 1-propanol: NH₄OH: water, 60:30:10. Areas corresponding to the standard spots and the origin, and blank areas, were scraped off and counted.

ACKNOWLEDGEMENT

We thank Dr. H. Weissbach (Roche Institute of Molecular Biology) for his advice as well as generous samples of ribosomes, Drs. E. Hamel and M. Cashel (National Institute of Child Health and Human Development) for assistance with binding assays and for samples of ribosomes, and Dr. H. Shichi (National Eye Institute) for use of the Cary 61 CD spectrometer.

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