THIOLATED BASES IN RIBOSOMAL RNA OF E. COLI

Rosalind I. Cotter and W.B. Gratzer
Medical Research Council Biophysics Unit,
King's College, Drury Lane, London, W.C.2

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Summary: Ribosomal RNA of E. coli contains some 16 4-thiouridylic acid residues per ribosome. These are not available for reaction in the 70S particle until it is dissociated into subunits. All the RNA and protein thiol groups then react with N-ethylmaleimide. Circular dichroism and melting profiles indicate that the 4-thiouridylic acid residues are in base-paired regions of the chain.

Implication of thiol groups in determining the association state of ribosomal subunits was suggested by the work of Miyazawa and Tamaoki (1), who reported that the thermal dissociation of ribosomes is mitigated and reversed by 2-mercaptoethanol. Moreover, they have also found (2) that p-chloromercuribenzoate causes 70S ribosomes to break down into 50S and 30S subunits, which will reassociate with recovery of biological activity on removal of the blocking group. Irreversible dissociation is brought about by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and to a lesser extent by N-ethylmaleimide (NEM). Miyazawa and Tamaoki (3) also give reasons for supposing that the thiol groups in question are directly involved in the interaction of the subunits. Partial elimination of thiol groups and its effect on the subunit interaction is related to the loss of biological activity (4-7).

The presence of thiol groups in an RNA was first reported by Lipsett (8), who identified 4-thiouracil as a prominent minor constituent of tRNA. Other thiolated nucleotides are present in low concentrations (9). We have investigated the

possibility that thiol groups might also be present as minor constituents of E. coli ribosomal RNA, and that these might be relevant to the sensitivity of the ribosomal structure to reagents specific for thiol groups.

Materials and Methods

Ribosomes from E. coli (nuclease-deficient strain MRE 600) were prepared according to Kurland (10) free of extraneous proteins and tRNA. RNA was prepared by the method of Robinson and Wade (11); it contained less than 0.5% protein as judged by the Folin reaction (12), and no tRNA at the level of detectability (ca 1%) by electrophoresis in 2.5% polyacrylamide gels using pyronine Y as stain (13).

DTNB (Eastman Kodak) was recrystallised from acetic acid and rRNA (5 mg/ml) or 4-thiouracil (42 μM) (Chemapol Chemical Co., Czechoslovakia) were treated with the reagent as described by Ellman (14). After incubation at 37° for 5 hours, absorbances were measured at 412 nm against blanks.

NEM-1-^{14}C was supplied by Schwarz Bioresearch Inc. in solution at 10 μC/ml and specific activity of 10.3 mC/mM. Unlabelled NEM was added as carrier to give a final concentration of 10 mM. Ribosomes or their subunits at 10 mg/ml in 0.01 M Tris, 0.06 M KCl, 30 mM or 0.5 mM $MgCl_2$, pH 7.0, were incubated with the reagent for 5 hours at 37°. Samples were then examined in the analytical ultracentrifuge using ultraviolet absorption optics to show that no degradation had occurred. To remove excess reagent the ribosomes were pelleted and resuspended several times, and then dialysed. For controls, ribosomes were allowed to react with unlabelled NEM, ^{14}C-NEM was added one hour before the end of the incubation, and the

radioactivity determined after the washing procedure. The free RNA was reacted at 5 mg/ml in the same way, excess reagent being removed by applying 0.5 ml samples to G-25 Sephadex columns (4). After determining the counts in the ribosomes, aliquots were dissociated into protein and RNA using LiCl-urea (15), and the components were counted separately. Samples were counted in a Nuclear-Chicago instrument with an efficiency for ^{14}C of 20%, Triton X-100 being used to ensure uniform spreading of samples. All absorption spectra and melting curves were measured on a Beckman DK-2A spectrophotometer and circular dichroism with a Jouan Mark II Dicrographe. Concentrations were based on a specific absorptivity, E (1 mg/ml; 1 cm) = 145 for ribosomes (16) and of 217.5 for RNA (17).

Results and Discussion

When 70S ribosomes are exposed to NEM, there is no significant incorporation of counts into the RNA. When, however, the magnesium concentration is lowered to 0.5 mM, counts are incorporated into the RNA of the (50S + 30S) dissociated ribosomes as well as into the protein (Table I). The RNA thiols are evidently therefore protected by the structure in the presence of a sufficient concentration of magnesium. In either case, assuming that NEM reacts only with thiol groups, there are 45 available thiols in the ribosomal proteins, which agrees with the half-cystine content by amino acid analysis (18).

The counts retained in the RNA correspond to 16 thiol groups per 70S particle, or about one in 300 nucleotides. Extracted ribosomal RNA gives the same value, indicating that essentially all thiolated nucleotides are available to the reagent in the 50S and 30S subunits. With DTNB, using the colour value found for the reaction with 4-thiouracil, the

Table I

Incorporation of ^{14}C-NEM* by Ribosomes in High and Low Mg^{2+}

	70S	50S+30S	Control
Total counts incorporated into ribosomes after washing(10 mg)	1100	1280	background
Counts in total protein after washing	490	500	background
Counts in RNA after washing	background	210	background

* Counts represent an average of several runs.

Total counts in incubation = 1.72×10^5 counts per minute.

Background counts 5-10 counts per minute.

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concentration of the thiolated oomponent is again one residue in 300. A further estimate of the concentration of 4-thiouridylic acid, assuming that this is indeed the thiolated component present, can be made directly from the ultraviolet absorption band at 335 nm. The absorption spectrum of rRNA in this region is shown in Fig. 1. With a value for the molar absorptivity of 4-thiouridylic acid of 15,000 (19) the absorbance at 335 nm would correspond to one residue in 350. However, the absorption band is significantly hypochromic (see below), and allowing for this and assuming an average residual hyperchromicity for the unstructured chain (20) of 4%, a value of one thiouracil in 300 residues is again obtained. The close identity of the absorption band with that of 4-thiouridylic acid in tRNA (8) in both the neutral and alkaline forms (Fig. 1)

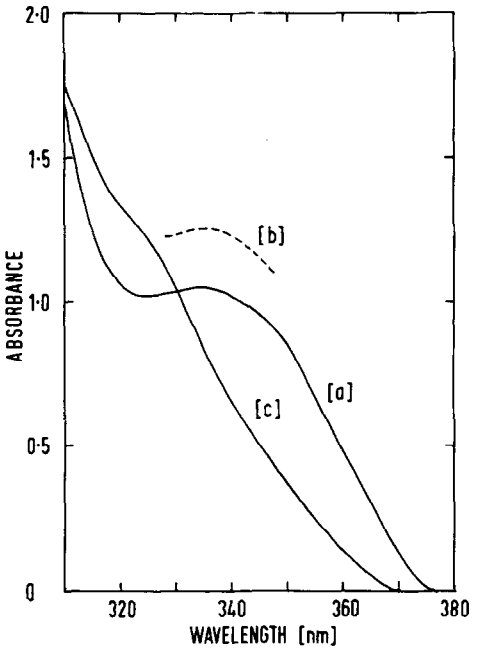

Fig. 1 Long-wavelength region of the ultraviolet absorption spectrum of E. coli ribosomal RNA. The RNA concentration is 8.75 mg/ml; solvent 0.05 M sodium chloride, 0.002 M cacodylate, (a) pH 7.0, 20°; (b) pH 7.0, 82°, in the presence of 2-mercaptoethanol; (c) adjusted to pH 10.5 with sodium hydroxide.

strongly suggests that this is indeed the chromophore which we are observing. Further conformation of its identity comes from circular dichroism measurements. The appearance of the Cotton effects associated with 4-thiouridylic acid is a function of the local structure and, as noted by Scott and Schofield (19), the elimination of structure by addition of a denaturing solvent is required to reduce the chromophore to an unperturbed state. Fig. 2 shows the circular dichroism of rRNA in aqueous buffer and in 80% dimethyl sulphoxide, which gives rise to a negative Cotton effect centered at about 332 nm, similar in shape and magnitude to that of the same chromophore in tRNA species. This transition is essentially reversible, the Cotton effect

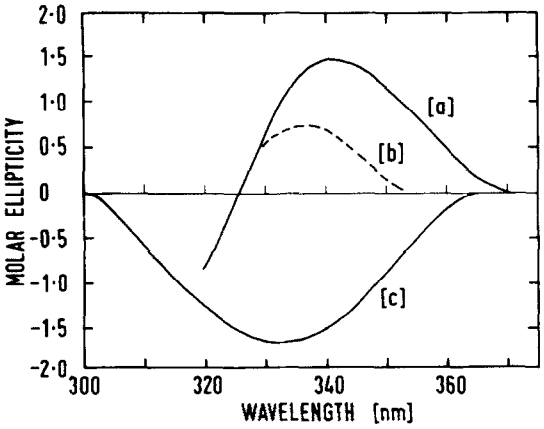

Fig. 2 Circular dichroism of E. coli ribosomal RNA in the near ultraviolet: (a) native RNA at pH 7.0, 20°; (b) adjusted to pH 10.5; (c) in 80% dimethyl sulphoxide.

reverting to positive on diluting out the dimethyl sulphoxide to 40%. In strongly alkaline solution, when the RNA is denatured and the chromophore ionised (Fig. 2), the Cotton effect again resembles that of 4-thiouridylic acid under the same conditions (21) down to the regions in which the principal Cotton effects of the RNA become predominant.

The inversion of the Cotton effect on denaturation (Fig. 2) suggests that it is involved in base-pairing, paired bases making up, according to our estimates (22) some 60% of the chain. Base-paired 4-thiouridylic acid in tRNA is hypochromic (8,23) and this is also true for rRNA. Fig. 3 shows temperature-absorbance profiles measured at 335 nm. In the absence of a reducing agent an initial absorbance increase occurs, centred at 40°, and is followed by a decrease; thereafter the melting is reversible, with a mid-point at 47°. Seno et al (23) have observed a rather similar effect in formylmethionine tRNA; moreover they have shown that the chromophore is not in itself heat-labile. We find, however, that if the melting is carried

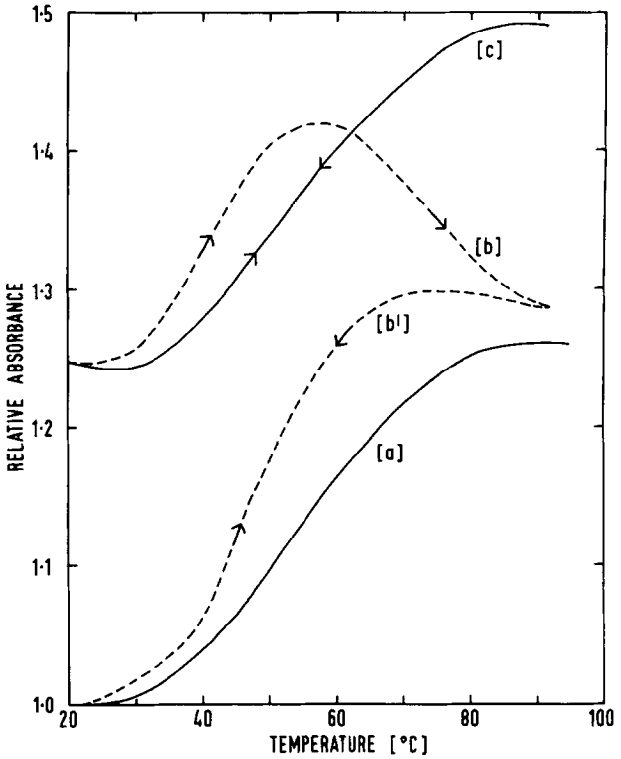

Fig. 3 Temperature-absorbance profiles of E. coli ribosomal RNA in 0.05 M sodium chloride, 0.002 M cacodylate, pH 7.0. (a) at 260 nm; (b) at 335 nm in the absence of reducing agent. The second and subsequent cycles are indicated by (b'); (c) at 335 nm in the presence of 0.05 M 2-mercaptoethanol. This curve is reversible. Melting curves were recorded automatically.

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out in the presence of 2-mercaptoethanol, the reversible profile shown in Fig. 3 is obtained. We conclude that in the absence of a reducing agent and in the presence of air, oxidation occurs, and disulphides are formed (cf. Lipsett (24)), the bases remaining in the paired state.

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