

Accessibility of phosphates in domain I of 23 S rRNA in the ribosomal 50 S subunit as detected by R_P phosphorothioates

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Abstract

Recent atomic models of ribosomal structure emphasize the need for new biochemical methods, suitable for fine-scale studies of ribosomal structure and function. We have used the phosphorothioate approach to probe iodine accessibility of 23 S rRNA domain I phosphates inside functional 50 S ribosomal subunits. Five percent of R_P isomers of nucleoside phosphorothioate were incorporated into *Thermus aquaticus* 23 S rRNA during in vitro transcription. Ribosomal large subunits were reconstituted from 23 S rRNA and 5 S rRNA transcripts and ribosomal large subunit proteins. The resulting particles sedimented as 50 S and were active in a peptide bond formation assay. Iodine-induced cleavage sites were determined for domain I of 23 S rRNA by reverse transcriptase-directed primer extension. Specific signals were detected at 360 positions, 80 of which were protected in reconstituted 50 S subunits. We argue that most observed protections are caused by shielding of phosphates by ribosomal proteins. The phosphorothioate approach can be extended to analyze dynamic structural changes during translation and the functional roles of individual chemical groups in rRNA.

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1. Introduction

The atomic structures of both ribosomal subunits have been determined [1–5]. This remarkable achievement makes it possible to study ribosome functioning at the atomic level by biochemical methods. Structural studies have strongly emphasized the importance of rRNA in the structure and function of the ribosome, including in the catalysis of peptide bond formation. The roles of individual nucleobases in rRNA have been analysed by site-directed mutagenesis and chemical probing methods [6]. The role of the sugar–phosphate backbone of rRNA is more difficult to analyze due to lack of proper methods. A promising tool to access this problem is the incorporation of phosphorothioate-substituted nucleotides into the sugar–phosphate backbone. Phosphorothioate substitutions can be readily analyzed using iodine-induced cleavage of the RNA backbone, which is specific to phosphorothioate bonds. This method allows the mapping of contact areas of the RNA

backbone by the footprinting approach. In addition, phosphorothioate substitutions have been utilized as chemical tags in combination with other ribonucleotide modifications for identifying the chemical groups that are important for RNA activity. The use of this approach, termed nucleotide analogue interference mapping has been reviewed in Ref. [7].

Reconstitution of functional 50 S subunits from in vitro transcribed 23 S rRNA has been reported for *Thermus aquaticus* [8] and for *Bacillus stearothermophilus* [9]. This method allows preparation of ribosomal subunits containing 23 S rRNA with artificially modified nucleotides.

Here, we report the reconstitution of functionally active 50 S ribosomal subunits with in vitro transcribed 23 S rRNA, containing R_P isomers of nucleoside phosphorothioates. We further use phosphorothioate-containing 50 S subunits to study the iodine accessibility of phosphate groups in the domain I of 23 S rRNA. Domain I has been found to be intimately involved in the large subunit assembly [10]. Deletions in the domain I have a dramatic effect on the ribosome assembly [10,11]. Ribosomal proteins L24 and L20, which are essential for large ribosomal subunit assembly, associate mostly with the domain I [2,5]. In the 70 S

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ribosome, the domain I is located on the solvent side of the 50 S subunit.

The iodine protection pattern of phosphates in domain I of 23 S rRNA of *T. aquaticus* is in good agreement with the structural data on 23 S rRNA–ribosomal protein contacts in *Deinococcus radiodurans* 50 S subunits, indicating that the shielding of phosphates in the ribosome is mostly due to rRNA–protein interactions.

2. Materials and methods

2.1. In vitro rRNA synthesis

T. aquaticus 23 S rRNA and 5 S rRNA were transcribed from plasmids kindly provided by A. Mankin [8]. Plasmids were cleaved for run-off transcription by *Bsp*119I and *Eco*RV, respectively [8]. rRNA was transcribed as described in Ref. [12] in a mixture containing 40 mM Tris pH=8, 5 mM DTT, 1 mM EDTA, 10 mM Mg(OAc)₂, 0.5 mM MnCl₂, 2 mM spermidine, 2 mM rNTP, 100 U/ml RNase inhibitor (Fermentas), 0.025 U/ml inorganic pyrophosphatase (USB), 40 µg/ml linearized rDNA template and T7 RNA polymerase. For the preparation of phosphorothioate transcripts, four reactions were performed where one rNTP was supplemented with 5% (0.1 mM) of the corresponding S_p rNTPαS (NEN). Transcriptions were carried out in 1-ml reaction volumes at 37 °C for 3 h. T7 RNAP over-expressing plasmid [13] was a kind gift of Dr. R. Sousa. T7 RNA polymerase was purified as described in Ref. [13].

2.2. Purification of rRNA transcripts

23 S rRNA transcripts were purified in 100-µl aliquots using 0.8 ml Sephacryl S-400 (Pharmacia) spin columns equilibrated with water. 5 S rRNA transcripts were similarly purified using a Sephadex G-50 (Pharmacia) matrix. Transcript concentrations after purification were 300–400 µg/ml.

2.3. Preparation of ribosomal 50 S subunits and TP50 proteins

T. aquaticus cells were purchased from the Center of Marine Biotechnology Institute, University of Maryland. *T. aquaticus* ribosomes were prepared and 50 S ribosomal subunits extracted using sucrose gradient centrifugation as described in Ref. [8]. TP50 was prepared with acetic acid extraction according to Ref. [14].

2.4. Reconstitution of *T. aquaticus* 50 S subunits

Reconstitution of *T. aquaticus* 50 S subunits using phosphorothioate-containing 23 S rRNAs was done as described in Ref. [8]. Reconstituted 50 S subunits were purified twice using Sephacryl S-200 (Pharmacia) spin

columns equilibrated with reconstitution buffer (20 mM Tris pH 7.4; 400 mM NH₄Cl; 6 mM spermidine; 5 mM β-mercaptoethanol) containing 40 mM MgCl₂ (Rec40). In parallel experiments, reconstituted 50 S were purified by sucrose gradient centrifugation. Ten to 20% sucrose gradient in 120 mM MgCl₂, 50 mM Tris pH 7.4, 100 mM NH₄Cl, 6 mM Spermidine, 6 mM β-mercaptoethanol was formed in a Beckmann Sw 28 rotor and centrifuged at $\omega^2 t = 2.79 \times 10^{11}$. Fractions containing 50 S were collected and ethanol precipitated. The concentration of 50 S subunits was determined by absorbency at 260 nm using the conversion coefficient 1 A₂₆₀ unit=37 pmol 50 S. The efficiency of reconstitution was assayed using the puromycin assay. Fifteen microliters of reconstituted 50 S in the reconstitution buffer, 1.5 pmol [³⁵S]f-Met-tRNA (10⁵ cpm) and 40 pmol puromycin were mixed in Rec20 buffer (20 mM MgCl₂) in a total volume of 50 µl, after which 30 µl methanol was added. Reactions were incubated for 1 h at 30 °C, then stopped with 10 µl 10 M NaOH for 15 min at 37 °C. Subsequently, 200 µl 1 M phosphate buffer pH 7.0 and 1 ml ethyl acetate were added, mixed by vortexing and centrifuged for 5 min. Five hundred microliters of the ethyl acetate phase (which contained [³⁵S]f-Met-puromycin) was collected and counted in a liquid scintillator counter. The minus puromycin experiment served as a negative control. The relative activity of reconstituted 50 S subunits was obtained by dividing the puromycin-accepting activity of the reconstituted 50 S by native 50 S subunits. Native 50 S subunit concentration used in the puromycin reaction was equimolar to the 23 S rRNA transcript, which was put into 15 µl of the reconstitution mix.

2.5. Iodine cleavage of modified 23 S rRNA

Iodine cleavage was carried out on reconstituted 50 S subunits in the Rec40 buffer. As controls, naked phosphorothioate-containing 23 S rRNAs were iodine treated. For modification interference experiments, 23 S rRNA was extracted from 50 S subunits after reconstitution and purification by sucrose gradient centrifugation, followed by iodine treatment. 50 S or 23 S rRNAs were treated with 10 mM I₂ (0.1 M I₂ stock solution was prepared in ethanol) for 1 min at 0 °C. The reactions were stopped with 50 mM β-mercaptoethanol. Cleaved RNAs were ethanol precipitated, dissolved in water and stored at –20 °C. 50 S subunits were subjected to rRNA extraction using 4.2 M guanidine thiocyanate and 4% Triton X-100 in water in the total volume of 1.2 ml. The solution was mixed vigorously for 20 min in an eppendorf shaker, followed by addition of 20 µl 50% silica suspension. For RNA binding, the suspension was gently mixed for 10 min and centrifuged at 6000 rpm for 10 s. The pellet was washed once with 1 ml 5 M guanidine thiocyanate and twice with 1 ml 50% ethanol. The pellet was then dried for 10 min at 37 °C and the RNA was eluted with 50 µl water at 42 °C for 15 min.

2.6. Primer extension

Iodine-induced cleavage sites were determined by primer extension using reverse transcriptase. Primer extension with [α - 32 P] dCTP labelling was done as in Ref. [15]. Two units of AMV (Seikagaku) or Superscript (Gibco) reverse transcriptase, 0.5–1 μ g of modified rRNA template and 1–5 pmol of primer were used per reaction. Primer extension products were separated by 7% urea–PAGE. The following primers were used: TA52- GGC TTA TCG CAG GTA GCC ACG; TA100- CCA TTC GGA CAT CCA GGG ATC; TA140- CCC GCA CAA AGG CGG TGA CCC GTG; TA201- GAT TTC TCT TTC CTC TCC TC; TA281- CGG CAT CTG AGG GCC CCA CGAC; TA402- CCA TGG CTC CAC GAA CCA CGG; TA441- CCC GGA GTA CTT AGC CTT AGG CGG; TA500- CCT CCC GGG GTT CTT TTC ACC; TA580- GGC TCA TGC TTC AAT AGG CAC GC.

3. Results

3.1. In vitro transcription of phosphorothioate-modified 23 S rRNA

The S_P (but not R_P) stereoisomer of α -phosphorothioate nucleoside triphosphates can be incorporated into RNA transcripts, accompanied with inversion into the R_P configuration [16]. The efficiency of nucleotide incorporation into RNA is not influenced by α -phosphorothioate substitution [16,17]. We performed separate transcription reactions from the 23 S rDNA template, where one of the four nucleoside triphosphates was present as a mixture of the unmodified compound with 5% of the corresponding α -phosphorothioate derivative. The yield of transcription reactions containing phosphorothioates was similar to the control reaction using unmodified nucleoside triphosphates, being 300–400 μ g of purified 23 S rRNA per milliliter of transcription reaction.

In preliminary experiments, different fractions of α -phosphorothioates in 23 S rRNA were tested for iodine cleavage. One percent of α -phosphorothioate was found to be the lower limit for specific signal detection by reverse transcriptase (data not shown). Throughout the study, 5% of α -phosphorothioates in 23 S rRNA were used.

3.2. Characterization of reconstituted 50 S subunits containing phosphorothioates in 23 S rRNA

50 S ribosomal subunits were reconstituted using phosphorothioate-containing transcripts of *T. aquaticus* 23 S rRNA, in vitro transcribed *T. aquaticus* 5 S rRNA and the large subunit protein fraction (TP-50) which was extracted from native 50 S subunits. In vitro transcribed unmodified 23 S rRNA was used in a control reconstitution. Reconstituted particles were characterized by sucrose gradient centrifugation. By comparing the sedimentation rates of

reconstituted particles and native 50 S subunits, it is possible to infer the compactness and the sedimentation coefficient of the reconstituted particles. Reconstituted particles form a single peak, which is similar to the native 50 S (Fig. 1). This implies native-like size and compactness for reconstituted large ribosomal subunits. Also, reconstituted subunits sediment at the same rate as native *T. aquaticus* 50 S subunits (Fig. 1). No free 23 S rRNA was detected by sucrose gradient analysis in the reconstitution reactions. Therefore, reconstituted phosphorothioate-containing large ribosomal subunits constitute an apparently homogenous population with the sedimentation coefficient 50 S. Iodine treatment does not change the sucrose gradient pattern and therefore the compactness or the sedimentation rate of the reconstituted 50 S subunits (Fig. 1). Larger absorbance at the top of the gradient of iodine-treated subunits is caused by β -mercaptoethanol, used to quench the iodine reaction.

Functional activity of the reconstituted ribosomal particles was assayed by the peptidyl transferase reaction where puromycin acts as the acceptor substrate and formyl-Met-tRNA as the donor substrate. When unmodified 23 S rRNA transcript was reconstituted, the peptidyl transferase activity of the resulting 50 S subunits was 14% compared to the native *T. aquaticus* 50 S subunits (Table 1). This is comparable to the results of Khaitovich et al. [8]. 23 S rRNA transcripts containing 5% α -thio-(A, C, G or U) were incorporated into 50 S particles and the corresponding particles exhibited 13–16% relative peptidyl transferase activity (Table 1). Thus, the presence of phosphorothioate substitutions at 5% of the corresponding nucleotide in the 23 S rRNA did not influence the functional activity of the reconstituted 50 S subunits.

3.3. Accessibility of 23 S rRNA domain I in 50 S ribosomal subunits

Accessibility of phosphates was determined in the reconstituted 50 S subunits and in the free 23 S rRNA by iodine-induced rRNA cleavage. 23 S rRNA samples containing 5% of a nucleotide phosphorothioate (e.g. AMP α S) were treated with iodine and subjected to reverse transcriptase-directed

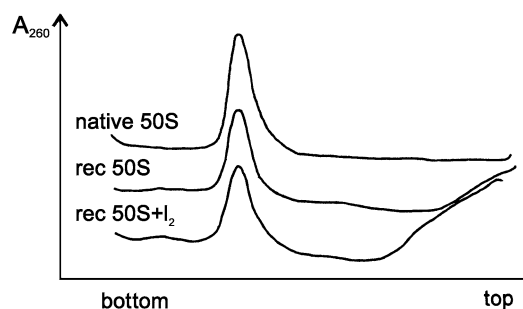


Fig. 1. Superimposed sucrose gradient profiles of native *T. aquaticus* 50 S subunits and reconstituted 50 S containing 5% U α S before (denoted rec 50S) and after (denoted rec 50S+I₂) iodine treatment. Y-axis shows absorbance at 260 nm, X-axis indicates bottom and top of the gradient.

Table 1

Relative peptidyl transferase activity of reconstituted *T. aquaticus* 50 S ribosomal subunits

23 S rRNA	Relative activity
5% rATP α S	0.14
5% rCTP α S	0.13
5% rGTP α S	0.14
5% rUTP α S	0.16
Unmodified 23 S rRNA	0.14
Native 50 S subunits	1.00

The puromycin reaction was carried out on aliquots of reconstituted 50 S particles, which were later used in iodine probing. Relative peptidyl transferase activity was calculated using native *T. aquaticus* 50 S activity as a standard. Unmodified 23 S rRNA means in vitro transcribed, reconstituted rRNA, which does not contain phosphorothioate modifications.

primer extension. For a negative control, substituted 23 S rRNAs were subjected to primer extension, omitting iodine treatment. Examples of cleavage patterns in two regions of 23 S rRNA are shown in Figs. 2 and 3. Primer extension stop sites were specific to the phosphorothioate nucleotides in the 23 S rRNA as nucleotide-specific signals were detected in all four thioate-substituted 23 S rRNA samples upon treatment with iodine. For reasons unknown to us, phosphorothioate-specific primer extension stops were shifted by one nucleotide toward the tops of the gels. At some positions, doubling of iodine-induced primer extension stops was observed where the “extra” band co-

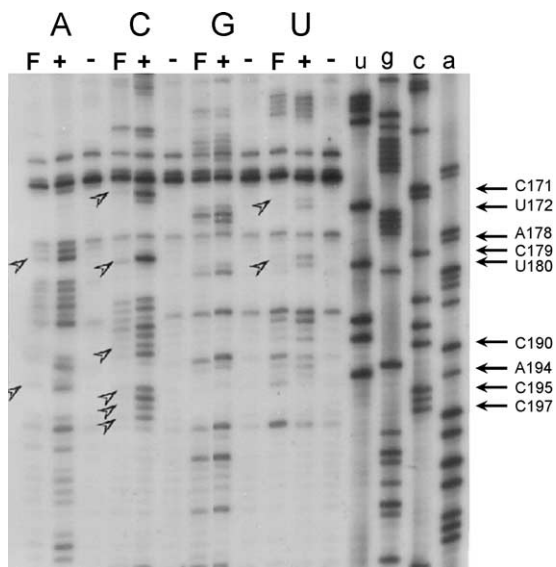


Fig. 2. Iodine-induced cleavage sites in the 23 S rRNA region 155–210. Autoradiography of a reverse transcriptase-directed primer extension, separated in a 7% urea polyacrylamide gel. Reconstituted 50 S subunits (lanes F) and denatured 23 S rRNAs (lanes +) were iodine treated, purified and subjected to primer extension. As a negative control, phosphorothioate-containing naked 23 S rRNA was submitted to primer extension omitting the iodine treatment. A, C, G and U denote 23 S rRNA transcripts into which the corresponding rNTP α S was incorporated; u, g, c and a denote DNA sequencing lanes. Positions, which are protected from iodine cleavage in the 50 S subunit, are indicated by arrowheads; corresponding positions in the sequencing lanes are indicated by arrows.

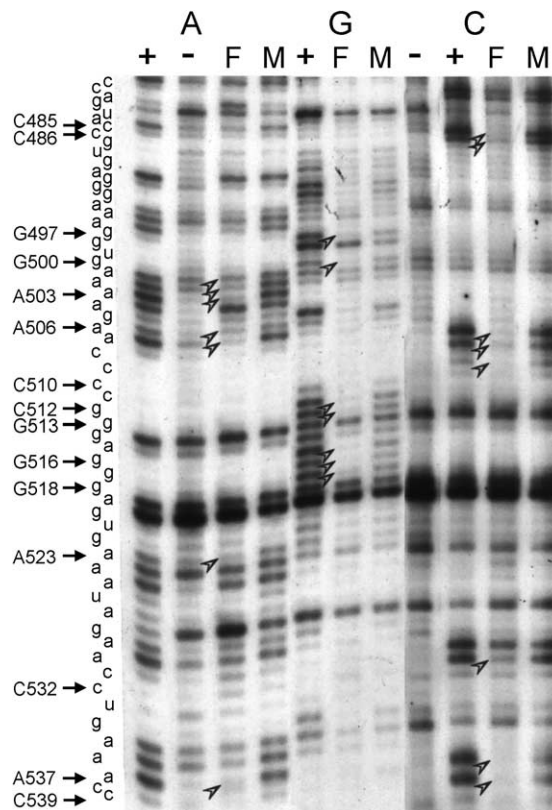


Fig. 3. Iodine-induced cleavage sites in the 23 S rRNA region 480–539. M lanes denote modification interference experiments where reconstituted 50 S subunits were purified from sucrose gradients and 23 S rRNA extracted before iodine treatment. Other annotations are the same as in Fig. 2.

migrated with the thioated nucleotide in the template (e.g. see Fig. 2, U180, U172).

A specific set of phosphate groups in 23 S rRNA was clearly less accessible to iodine in the 50 S subunit as compared to the free rRNA. These phosphates were taken to be protected when at least a two-fold, reproducible reduction in iodine-induced rRNA cleavage occurred. We analyzed accessibility of phosphates 1–580 of 23 S rRNA corresponding to structural domain I in the 50 S ribosomal subunit. Two hundred eighty positions were accessible to iodine in the reconstituted 50 S (marked in blue in Fig. 4) and 80 positions were protected (marked in red in Fig. 4).

The apparent protection of specific phosphates could be caused by functional interference of phosphorothioates during ribosome reconstitution: if a modified nucleotide at a particular position prevents the 23 S rRNA molecule to be incorporated into 50 S subunit, the position would show up as an apparent protection site in the primer extension gel. To test this possibility, we analyzed modification interference at two L24 binding regions, at positions 65–160 and 470–540 [18]. Ribosomal protein L24 is known to be essential for early events during ribosomal large subunit assembly [18]. Modifications at the L24 binding site could potentially disrupt L24 binding to 23 S rRNA and thereby assembly of the 50 S subunit, resulting in modification interference to

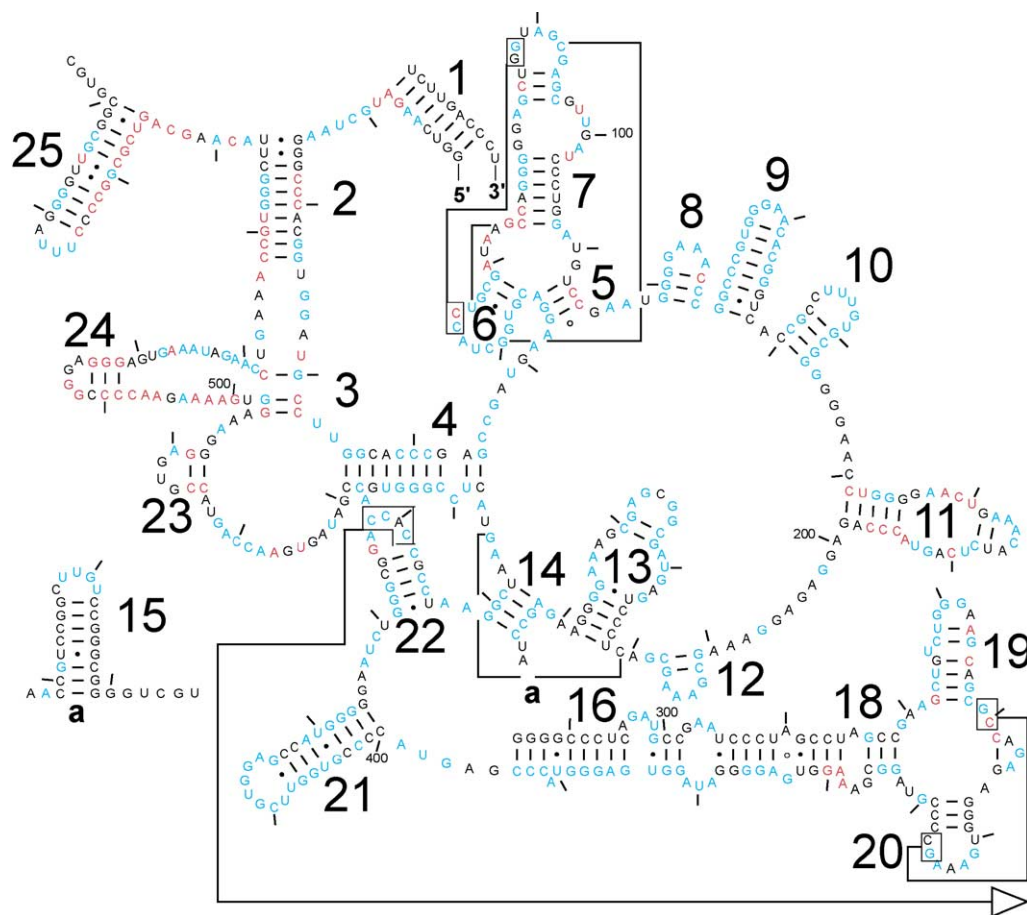


Fig. 4. Summary of protected and accessible positions in domain I of *T. aquaticus* 23 S rRNA as determined by iodine cleavage of reconstituted transcripts. Protected positions are red and accessible positions blue. Positions whose accessibility could not be determined are shown in black.

50 S assembly. We compared iodine accessibility of phosphates in the unreconstituted 23 S rRNA (for the 470–540 region, see Fig. 3, + lanes) with the 23 S rRNA extracted from reconstituted 50 S subunits and subsequently treated with iodine (Fig. 3, M lanes). Results of the footprinting experiment for the region C479–C540 are shown on F lanes in Fig. 3. Iodine-induced cleavage at 22 positions was reduced in the 50 S subunits indicating that these phosphates are protected. Comparison of the 23 S rRNA primer extension pattern before reconstitution (+ lane) to that extracted from 50 S subunits (lane M) reveals no difference in band intensities. This result clearly shows that phosphorothioate modifications in the region C479–C540 of 23 S rRNA do not interfere with the incorporation of 23 S rRNA into 50 S subunits. A similar result was obtained for the region C65–G160 (data not shown).

4. Discussion

We have developed a footprinting technique which is suitable for large RNPs, such as the ribosome. Phosphorothioate-substituted nucleotides were incorporated into 23 S rRNA, reconstituted into 50 S ribosomal subunits, probed

with iodine, and analyzed by reverse transcriptase-directed primer extension. This method allows the analysis of both single-stranded and double-stranded regions of RNA. Also, the phosphorothioate-substitution method probes the accessibility of the rRNA backbone, thus being complementary to traditional ribosomal footprinting methods, which probe the bases of rRNA. Implementing the phosphorothioate approach into large ribosomal RNA studies presents some technical complications. The end-labelling method used to detect phosphorothioate-containing positions in small or medium size RNAs cannot be used on high-molecular-weight rRNAs. We have used reverse transcriptase-directed primer extension for detection of iodine cleavage sites.

We were able to unambiguously ascertain the protection status of 360 phosphates (62%) of 23 S rRNA domain I (Fig. 4). As expected, both single-stranded and structured RNA regions were generally amenable to analysis (Fig. 4). Primer extension reactions from substituted, but not iodine-treated transcripts, resulted in numerous non-specific stops common to all lanes (Figs. 2 and 3). Such general stop sites could be caused by the secondary structure and/or degradation of the rRNA template. The positions that were excluded from analysis because of universal primer extension stops or weak specific signal, are shown in black in Fig. 4. Another

reason for excluding some positions from analysis was because variable results were found at a few specific positions. An example is position G175 that is apparently protected in Fig. 2 but was found to be accessible in parallel experiments. Ten specific protections are indicated by arrowheads in Fig. 2. The relative weakness of thio-A-substituted positions in Fig. 2 is misleading. In parallel experiments, only A178 and A194 showed up as protected in a reproducible way. In addition, positions C469, A473, G476, A496 and G505 gave conflicting protection data in parallel experiments when different 50 S preparations were used (Fig. 2 and not shown). These positions (as is G175) are located in 23 S rRNA regions which are enriched in protections (Fig. 4) and are at or near several r-protein binding sites (see discussion below). Therefore, it is likely that variable protection data at aforementioned positions points to some structural variability in different preparations of reconstituted 50 S subunits.

Five percent incorporation of ATP α S, CTP α S, GTP α S or UTP α S should lead to about 32, 39, 53 or 22 of the corresponding nucleotides to be thio-substituted per 23 S rRNA molecule. This begs the question of the functionality of the reconstituted subunits. However, phosphorothioate substitutions did not influence the peptidyl transferase activities of reconstituted 50 S (Table 1), yield of reconstitution or compactness of reconstituted subunits as determined by sucrose gradient centrifugation (Fig. 1 and not shown). Therefore, phosphorothioate-containing 50 S subunits retain their structure and activity.

In addition to specific RNA backbone cleavages at thioated positions, the iodine treatment could oxidize cysteins and methionines of r-proteins and thus potentially disrupt the 50 S particles. However, because iodine treatment does not change the sedimentation profiles and there-

fore the compactness of reconstituted 50 S (Fig. 1), we conclude that iodine treatment leaves the higher ordered structure of 50 S subunits intact.

The recently published crystal structure of the *D. radiodurans* 50 S subunit [5] allowed us to model our protection data from *T. aquaticus* (see Fig. 4) into the 50 S structure of *D. radiodurans* (Fig. 5). *D. radiodurans* and *T. aquaticus* are phylogenetically related, belonging to the same phylum [19]. It should be noted that only α -carbons of the r-proteins were determined by Harms et al. [5]. Therefore, coordinates of protein side chains are unavailable to us, limiting the scope of interpretation, which can be obtained from our modelling endeavour. However, tentative attribution of the observed iodine protections to r-proteins and other structural elements of the 50 S is possible. Comparison of protections, modelled with (Fig. 5A) or without r-proteins (Fig. 5B), leads to two findings. Firstly, protected positions (red) tend to be clustered in the secondary structure (Fig. 4) and even more so in the three-dimensional structure, leaving a large part of the domain I devoid of protections (Fig. 5B). Secondly, most protected positions are positioned close to r-proteins (Fig. 5A). We take the presence of a protein α -carbon within 11 Å distance from the protected phosphate as suggestive of the protection being caused by the corresponding protein. Validity of this assumption is supported by the analysis of placement of iodine-generated r-protein footprints in ribosomal crystal structures by Whirl-Carrillo et al. [20] where most iodine protections fell closer than 15 Å to the nearest protein α -carbon. According to our analysis, the base of helix 1 is protected by L13. Protections in helix 2 are caused by L20, L22 and L32. Helix 3 is contacted from different sides by L20 and L24. Protections in the 23 S rRNA region 70–76 are in proximity of L29. Protections in helix 7 are caused by L24, in helix 11 by L34, in helix 19 by

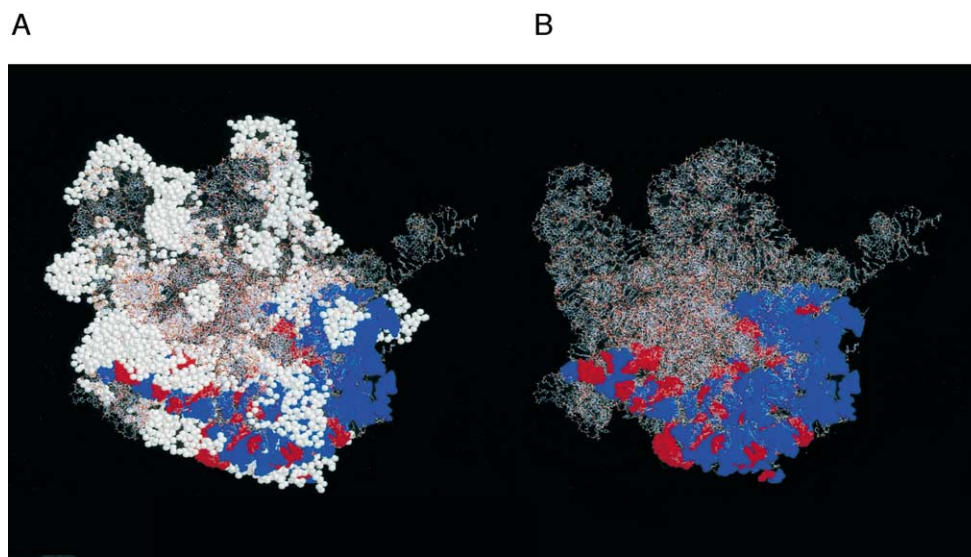


Fig. 5. Protection data, modelled into the structure of *D. radiodurans* 50 S [5]. A solvent-side view of the 50 S is presented using RasWin Molecular Graphics. Protected nucleotides are shown in red space fill and accessible nucleotides in blue space fill. (A) α -Carbons of r-proteins are shown in white space fill. (B) r-Proteins are omitted.

L4 and L24, in helix 23 by L34 and L23, in helix 24 by L22 and L24, in the base of helix 25 by L13 and L20.

Some protections in helices 3 (positions 32 and 532) and 11 (positions 171, 172, 179, 190, 194 and 195) are likely caused by shielding by rRNA. The upper half of helix 25 (positions 561–565) and protected positions 368–370 in helix 18 are located on the surface of *D. radiodurans* 50 S subunit and should not contact any r-proteins. We conclude that the most common reason for iodine protection of rRNA backbone is shielding by protein.

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