PHOTOINCORPORATION OF TETRACYCLINE INTO *ESCHERICHIA COLI* RIBOSOMES

Identification of labeled proteins and functional consequences

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1. Introduction

The *E. coli* ribosome is currently the object of a great variety of studies which seek to define its function in terms of its structure. Photoaffinity labeling has proven to be a valuable tool in this regard [1]. We are currently using this technique to localize antibiotic binding sites on the ribosome. Our basic premise is that the function which a given antibiotic inhibits is likely to be carried out in the general vicinity of where the antibiotic binds, so that localization of the antibiotic binding site provides presumptive evidence for localization of the functional site.

It has been shown that native puromycin photoincorporates into specific sites on the *E. coli* ribosomes [2]. Taking note of the intrinsic photolability of tetracycline [3,4], we decided to investigate the possible photoincorporation of native [^{3}H]TC into *E. coli* ribosomes. In this communication we:

(i) Show that such incorporation takes place;
(ii) Identify the major labeled ribosomal proteins as being S18 and S4, and to a lesser extent, S7, S13 and S14;
(iii) Present evidence that the observed incorporation is related to the functional properties of TC.

This article represents the efforts of two groups working independently (R.A.G., B.S.C. and W.A.S.; B.A.W. and T.R.T.). We learned of each other's results as separate manuscripts were being prepared for publication. Because the two sets of results are in close agreement where they overlap directly, and are otherwise complementary, we present them jointly in this publication.

Abbreviations: TLC, thin layer chromatography; TC, tetracycline; PAGE, polyacrylamide gel electrophoresis

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2. Experimental

Tetracycline hydrochloride was purchased from Boehringer Mannheim or Sigma. [7-^{3}H]Tetracycline was purchased from New England Nuclear or Amersham and its purity was verified by TLC in three solvent systems. Analytical TLC was performed with Brinkmann silica gel G/UV_{254} plastic backed plates which were dipped in 0.1 M EDTA just before use.
Solvent system (A): 1 M NH_4Cl–acetonitrile (7:3), R_F-tetracycline = 0.56;
Solvent system (B): ethanol–water (1:1), R_F = 0.45;
Solvent system (C): *n*-butanol saturated with 5% citric acid, R_F = 0.1.

All TC solutions were stored dark and frozen and were replaced frequently because the drug undergoes both thermal and photochemical degradation.

70 S ribosomes were prepared from both *E. coli* MRE600 [5] and *E. coli* Q13 [6] as described. Large scale preparations of 30 S and 50 S subunits were obtained by zonal centrifugation in a Beckman Ti 15 rotor essentially as in [7]. Small quantities of subunits were prepared from 70 S ribosomes labeled with TC by sucrose gradient centrifugation in a swinging bucket rotor. Phe-$tRNA^{Phe}$ was prepared as in [8].

Photolysis experiments were performed using either a lamp assembly containing two 15 W self-

filtering, low pressure mercury vapor tubes having an output concentrated at 366 nm, or a Rayonet lamp assembly using RPR 3500 Å lamps having maximal output at 350 nm. When the first lamps were used, samples were contained in the wells of plastic tissue culture clusters (Costar) at ~10 cm from the lamps. Potassium oxalate actinometry [9] showed the incident dose to be 3.9×10^{16} quanta . s^{-1} . ml^{-1}. With the second lamps, samples were irradiated in vertical tubes as in [2] with an incident dose of 11×10^{16} quanta . s^{-1}. ml^{-1}. All photolyses were performed in standard TMK buffer (50 mM Tris, 10 mM $MgCl_2$ or $MgOAc_2$, 100–150 mM KCl (pH 7.5–7.6)) at 4°C. One- and two-dimensional PAGE were performed as described. Specific immunoprecipitation was performed by a sandwich technique [22] as in [10].

3. Results

When [^{3}H]TC is irradiated with either the RPR 3500 Å or the 366 nm lamps in the presence of *E. coli* ribosomes, it becomes irreversibly bound to the ribosome. Table 1 shows the RNA–protein and 30 S–50 S distribution of the irreversibly bound [^{3}H]TC. In both experiments shown incorporation occurs primarily into ribosomal protein, with the 30 S subunit being labeled to a greater extent than the 50 S. Similar results were obtained in a variety of other experiments. The small, quantitative differences between the two experiments seen in table 1 probably arise from procedural differences in the two experiments, rather than as a result of the different levels of TC exployed.

Several experiments were carried out to clarify aspects of the photochemical reaction leading to attachment, as shown in table 2. Heating the ribosomes at 37°C for 30 min, which results in an enhancement of functional assays [11], results in an increased uptake, possibly because a larger fraction of the ribosomes are in the 'correct' configuration to accept the binding of TC. The reaction is dependent upon the presence of Mg^{2+} as the presence of EDTA abolishes the covalent uptake of TC. F-Aminobenzoic acid, a free radical scavenger, reduces the uptake of TC suggesting that the process may proceed by a free radical intermediate. The possibility that a photoreduction is involved in the mechanism of attachment is intimated by the finding that isopropanol, a good hydrogen donor, greatly stimulates the covalent incorporation of drug into the 70 S ribosomes. Finally, isolated 30 S subunits show enhanced total incorporation as compared with 70 S ribosomes.

Table 1
Distribution of covalently incorporated [^{3}H]tetracycline into *E. coli* ribosomes

	% Incorporated TC	
	Expt. A	Expt. B
30 S Subunit	69	59
50 S Subunit	31	41
Protein fraction	96	89
RNA fraction	4	11

Expt. A: MRE 600 70 S ribosomes (21 μM) were irradiated in the presence of [^{3}H]TC (20 μM) for 2 h with the 366 nm lamps. To separate the subunits, 70 S ribosomes were dialyzed against a low [Mg^{2+}] buffer (50 mM Tris, 1 mM Mg-acetate, 150 mM KCl (pH 7.5)) for several hours and layered on a 10–30% sucrose gradient. The resulting centrifugation was for 17 h at 21 000 rev./min in a Beckman SW 40Ti rotor. Ribosomal RNA and protein fractions were obtained by adding an equal volume of 8 M urea and 4 M LiCl and incubating ≥24 h at 4°C. The rRNA precipitate was separated from the protein-containing supernatant by centrifugation. 100% incorporation corresponded to 7300 cpm for RNA/protein distribution and 1600 cpm for subunit distribution

Expt. B: Q13 70 S ribosomes (2.6 μM) were irradiated in the presence of [^{3}H]TC (50 μM) for 1 h with RPR-3500 Å lamps. After photolysis, ribosomes were precipitated by addition of 2 vol. ethanol, redissolved in buffer and ethanol-precipitated again, and separated into subunits by centrifugation through a 5–20% sucrose gradient in a high salt buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 50 mM KCl, 400 mM NaCl). The subunits were collected as in [6]. Protein was obtained by Mg^{2+}–acetic acid extraction followed by acetone precipitation [20]. RNA was obtained by phenol extraction followed by repeated ethanol precipitation of the water layer. 100% incorporation corresponded to 6200 cpm for RNA/protein distribution and 1.5×10^5 cpm for subunit distribution.

The identities of the major labeled ribosomal proteins have been determined by one- and two-dimensional PAGE and by specific immunoprecipitation analyses. Fig.1. shows the distribution of ^{3}H-radioactivity on one-dimensional PAGE analyses of labeled 30 S and 50 S proteins. The 30 S gel shows two major regions of labeling. One peak of radioactivity falls between the position of proteins S14 and S18 while another broad area of radioactivity spans the positions of proteins S3, S4, S5, S6, S7 and S8. The one-dimensional gel of [^{3}H]TC labeled 50 S protein

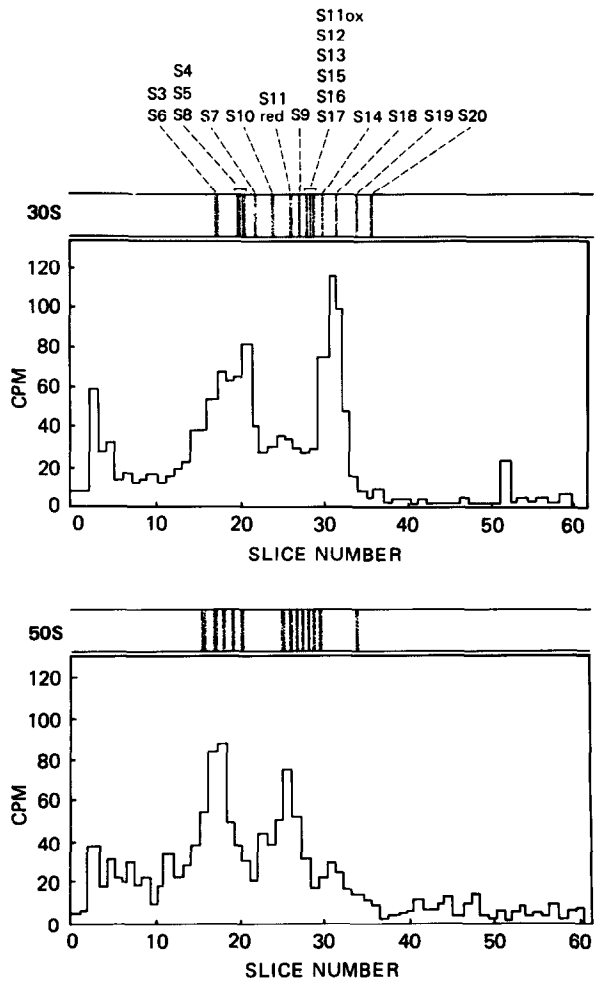

Fig.1. One-dimensional PAGE patterns of proteins from ribosomal subunits derived from 70 S ribosomes labeled with [^{3}H]tetracycline. Irradiation conditions and procedures for subunit and protein isolation were as in table 1, expt. B. In the absence of irradiation, essentially no radioactivity comigrated with ribosomal proteins.

shows two regions of labeling, both occurring in protein-dense regions of the gel.

Two-dimensional PAGE analyses were conducted on proteins isolated from the following species which had been irradiated with [^{3}H]TC: 70 S ribosomes, 30 S subunits irradiated directly, and 30 S and 50 S subunits isolated from labeled 70 S ribosomes. Quantitatively, 30 S subunits labeled directly with [^{3}H]TC show considerably higher incorporation levels than 30 S subunits isolated from labeled 70 S ribosomes (see table 2), but qualitatively, the observed labeling patterns are very similar. Also,

Table 2
The covalent attachment of tetracycline to 70 S ribosomes under various conditions

Condition	% Control TC incorporated
1. 70 S Subunit alone, control	100
2. 70 S, Heat-activated, 37°C, 30 min	147
3. 70 S + 10 mM EDTA	8
4. 70 S + 2.5% Isopropanol	250
5. 70 S + 15 mM *p*-Aminobenzoic acid	33
6. 30 S Subunit alone, relative to an equal no. mol 70S ribosomes	300–500

MRE 600 70 S ribosomes (15 μM) were irradiated at 366 nm in the presence of [^{3}H]TC (20 μM) for 2 h. Following irradiation aliquots were removed, precipitated with 7.5% trichloroacetic acid and collected on Whatman glass fiber filters (GF/A). The filters were then washed with 2.5% trichloroacetic acid, dried and counted in New England Nuclear formula 963 cocktail in a Beckman LS7000 scintillation counter. 100% incorporation corresponded in different experiments to 950–2178 cpm

labeling patterns of 30 S protein are similar over the (1.25–300 μM) TC used in the photoincorporation experiments. Two major regions of radioactivity are associated with 30 S protein. The more highly radioactive region does not coincide with protein stain, but rather falls in an area bound by proteins S13, S14, S15/16, S17, S18 and S19. The results of a typical analysis are shown in fig.2. The second major area of radioactivity is seen in the region of proteins S3, S4, S5 and S7. By contrast, two-dimensional PAGE analysis of proteins from 50 S subunits isolated from labeled 70 S ribosomes shows no major regions of radioactivity, so that it is probable that the apparent peaks of radioactivity seen in fig.1b reflect labeling of several proteins, with each being labeled to only a small extent. Finally, two-dimensional PAGE analysis of total 70 S protein shows the expected overlap of the patterns seen for 30 S and 50 S proteins separately.

Specific immunoprecipitation analysis of labeled 30 S protein provides definitive identification of the proteins which are the major sites of TC incorporation. As can be seen from table 3, S18 and S4 are labeled to the highest extents, and significant labeling is also seen in S14 and perhaps S7 and S13. These results are in good accord with the two-dimensional PAGE results presented above, if it is assumed that the major peak of radioactivity in fig.2 reflects labeled S18. Such an assumption requires that, with

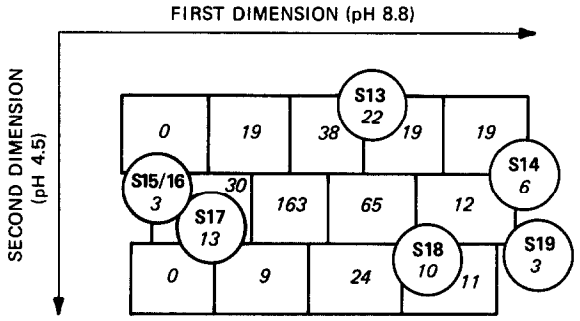

Fig.2. Area of highest radioactivity on two-dimensional PAGE of [^{3}H]tetracycline-labeled 30 S protein. Following electrophoresis, the gel slab was overlayed with a plastic sheet. Areas of protein staining were circled on the sheet, and areas not staining were divided into a grid, as shown. Portions of the gel corresponding to the delineated areas were excised and counted for radioactivity as in [6]. The drawing is from a xerox copy of the plastic sheet. S numbers (bold-face) refer to 30 S proteins. Italicized numbers are radioactivity in cpm. Experimental conditions: 7 μM 30 S subunits (Q13); 50 μM [^{3}H]TC. Photolysis was for 1 h with the RPR-3500 A lamps. Protein was obtained as in table 1, expt. B.

respect to native S18, labeled S18 be strongly retarded in the first dimension of electrophoresis but only weakly retarded in the second. This assumption is totally reasonable, since TC is an anion at pH 8.8 (first dimension) and a neutral zwitterion at pH 4.5 (second dimension) [12].

Two experiments were performed to examine the question as to how the labeling reaction relates to the inhibitory properties of TC on ribosome function. Both experiments were designed so that measured ribosomal activity was proportional to the concentration of added ribosomes. In the first, aliquots were taken as a function of time from a solution of ribosomes and [^{3}H]TC undergoing irradiation, and analyzed for total TC incorporation into ribosomes and activity in poly(U)-dependent polymerization of phenylalanine (fig.3). In the second experiment, the results of which are summarized in table 4, 30 S subunits were irradiated separately with [^{3}H]TC, combined with native 50 S subunits under conditions favoring 70 S ribosome formation, and the resulting hybrid ribosomes were examined for their ability to bind [^{14}C]Phe-tRNA^{Phe} and for TC incorporation. The same procedure was also employed with 9-NO_2 sanscycline, a structural analogue of TC which is a weaker (~5-fold) inhibitor of ribosomal function (R. A. Goldman, N. Zuño and B. S. Cooperman,

Table 3
Immunoprecipitation of [^{3}H]tetracycline-labeled 30 S proteins (% precipitated)[a]

Antiserum to protein	30 S isolated from labeled 70 S[b]	30 S labeled directly[c]
S3	2 ± 2	4
S4	23 ± 2	26
S5	4 ± 3	6
S7	11 ± 1	7
S13	9 ± 1	7
S14	12 ± 3	15
S15	−2 ± 1	–
S16	3 ± 1	–
S17	5 ± 1	–
S18	36 ± 3	25
S19	3 ± 1	4

[a] Results are expressed as the % of total radioactivity found in the sandwich precipitate after subtraction for non-specific occlusion of labeled protein (measured using a preimmune serum control sample). This blank averaged ~12% of the total radioactivity, and accounts for the lack of precision in determination of low labeling values. ~2000 counts were used for each determination

[b] Photolysis conditions and subunit and protein isolation are as in table 1, expt. B

[c] Photolysis conditions and protein isolation were as in fig.2.

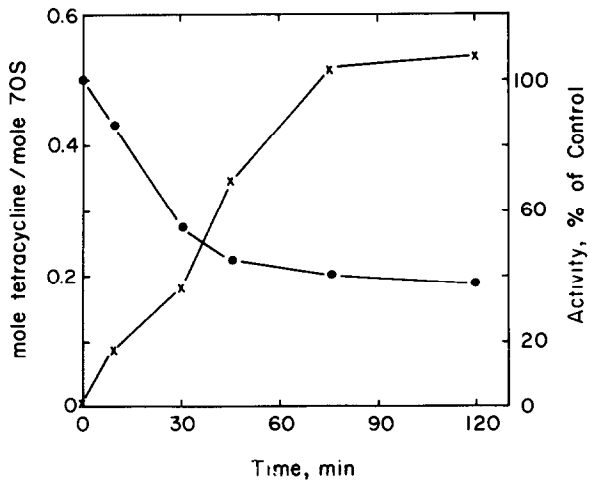

Fig.3. Correlation of [^{3}H]tetracycline incorporation and loss of polyphenylalanine synthesis activity. 70 S ribosomes (MRE 600, 20 μM) were irradiated in the presence of [^{3}H]TC (12.5 μM) with the 366 nm lamps. At the indicated times, aliquots were removed and analyzed for [^{3}H]TC incorporation and poly(U)-dependent poly(Phe) synthesis activity [21].

unpublished), although here incorporation was not measured since radioactive 9-NO_2 sanscycline was not available.

The results of these two experiments show that photoincorporation of TC accompanies ribosome inactivation. Analysis of the data in fig.3 shows that inactivation of polyphenylalanine synthesis correlates with the incorporation of 1 TC/ribosome. Similarly, from table 4 incorporation of 0.52 TC/ribosome correlates with a decrease in Phe-tRNA^{Phe} binding to 63% of the control sample, and this represents ~60% of the total expected response of this assay to TC. (In the absence of irradiation, saturating TC concentrations decrease Phe-tRNA^{Phe} binding to 35–40% that of the control sample, (R. A. Goldman, N. Zuño and B. S. Cooperman, unpublished)). A comparison of the results obtained on photolysis in the presence of either TC or 9-NO_2 sanscycline (table 4) suggests a correlation between the extent of photoinduced inactivation of ribosomal Phe-tRNA^{Phe} binding activity and antibiotic potency.

4. Discussion.

There is good evidence that the inhibitory effect of TC on cell-free protein synthesis arises predominantly from its inhibition of aminoacyl-tRNA binding to the A site [13]. Tetracycline appears to have a single tight-binding site on the ribosome as well as a multitude of weaker ones. Although the point is disputed in the literature [5,14], most of the evidence suggests that inhibition is correlated with tight-site binding of TC [14,15]. The results presented in fig.3 and table 4 are consistent with the proposition that the observed photoinduced inactivation of ribosomes is a direct consequence of the incorporation of 1 TC/ribosome. If the inhibitory properties of TC are properly attributed to tight-site binding, a further implication would be that incorporation is taking place into this site, and that portions of proteins S4 and S18 are accessible from it. It is notable in this regard that proteins S4 and S18 are also the principle targets for covalent reaction with the initiation codon analogue 5′-(4-(bromo-acetamido-phenylphospho)-AUG. [16].

While these ideas are certainly attractive, our results fall short of proving them to be true. It is

Table 4
Correlation of [^{3}H]tetracycline incorporation and loss of Phe-tRNA^{Phe} binding

Antibiotic	Irradiation at 350 nm	Antibiotic irreversibly bound/ribosomes	Relative poly(U)-dependent Phe-tRNA^{Phe} binding[a]
None	+	–	1.00
[^{3}H]TC, 50 μM	–	0.00	1.04
[^{3}H]TC, 50 μM	+	0.52	0.63
9-NO Sanscycline, 50 μM	+	n.d.	0.86

[a] In the absence of photolysis, addition of saturating TC (100 μM) decreases Phe-tRNA^{Phe} binding to 0.35–0.40 of the value obtained in the absence of TC

Isolated 30 S subunits were irradiated with [^{3}H]tetracycline, using conditions as in fig.2. Following photolysis, the subunits were ethanol-precipitated 4 times to remove unbound TC, and 0.33 A_{260} units were combined with 0.67 A_{260} units of untreated 50 S subunits in 100 μl of tRNA binding buffer (50 mM Tris–HCl (pH 7.6), 20 mM $MgCl_2$, 100 mM KCl, 6 mM β-mercaptoethanol, 240 μg poly(U)/ml). Samples were pre-incubated at 37°C for 10 min, [^{14}C]Phe-tRNA^{Phe} (6 pmol, 5000 cpm) was added, and incubation at 37°C was continued for an additional 10 min before filtering through Millipore HAWP 02500 filters (0.45 μm poresize). Samples were filtered immediately after 37°C incubation. Filters were dried and counted as in [6]. Relative poly(U)-independent Phe-tRNA binding of 0.14 was subtracted from each of the observed values, giving the relative poly(U)-dependent Phe-tRNA^{Phe} binding values shown. [^{3}H]Tetracycline irreversibly bound ribosome was determined from [^{3}H]cpm retained on the filter

possible that TC causes a photodynamic inactivation of ribosomes in a process independent of the covalent attachment. For example, *E. coli* ribosomes are readily photoinactivated by methylene blue in the presence of O_2 [17] and tetracycline has been previously shown to photoinactivate virus particles [18]. The lower inactivation seen with 9-NO_2 sanscycline (table 4), which should also be expected to give rise to photodynamic effects, argues against this possibility. Likewise one would not expect irreversible incorporation of 1/TC/ribosome to correlate with inactivation if photodynamic action were causing loss of function, although it is possible that the correlation is fortuitious. An additional consideration is that preparations of *E. coli* ribosomes are not conformationally homogeneous so that not every 70 S ribosome is active or in the same state. Our calculations assume that all of the ribosomal subpopulations are labeled to the same extent, but this need not be the case. In particular, it is possible that 'active' ribosomes are labeled to a higher extent then the general population, in which case inactivation would correlate with incorporation of >1 TC/ribosome. Finally, even if photoincorporation of TC were responsible for photoinactivation, the principal incorporation targets, proteins S4 and S18, might have nothing to do with tight-site TC binding. The loss of ribosomal function might simply arise as a consequence of modification. Results with *N*-ethylmaleimide are particularly relevant in this regard, since treatment of 30 S subunits with this reagent also gives S18 as the major labeled protein and leads to inhibition of both ribosomal tRNA binding and ribosome-dependent poly(Phe) synthesis [19].

In summary, we have shown that tetracycline can be photoincorporated into 70 S ribosomes, that S4 and S18 are the principal proteins labeled, and that loss of ribosomal activity accompanies photoincorporation. However, further experimentation will be needed before definitive conclusions can be reached on the relationships between tetracycline photoincorporation, ribosome inactivation, and the putative tight tetracycline-binding site.

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