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Functional Modification of 16S Ribosomal RNA by Kethoxal

(30S ribosomal subunits/tRNA binding site/guanine residues/reconstitution/protein synthesis)

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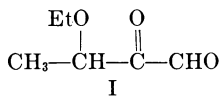
ABSTRACT **Kethoxal reacts with 30S ribosomal subunits to give totally inactive particles, as measured by *in vitro* protein synthesis. It is postulated that functional modification occurs at the binding site for transfer RNA since (*a*) loss of specific binding of transfer RNA, but not binding of messenger RNA, is simultaneous with loss of protein synthesis, and (*b*) loss of activity is inhibited by bound transfer RNA. By means of *in vitro* reconstitution and labeling techniques, it is found that loss of transfer RNA-binding activity is correlated with the modification of six or seven guanine residues in 16S RNA.**

An increasing number of chemical and genetic studies have been reported that bear on our understanding of the specific functions of various ribosomal proteins (see *Nature New Biol.*, **237**, 65, 1972). Apart from those dealing with the role of RNA in ribosome assembly (1–4), similar studies with ribosomal RNA have been conspicuously few. With ribosomal RNA comprising two-thirds of the mass of a bacterial ribosome (5), it should not be surprising to learn that it plays an active functional part in protein synthesis in bacteria. In an effort to obtain some clue regarding the functional role of 16S RNA, we have attempted to chemically modify it in such a way as to bring about an alteration in ribosomal function that might, at least in principle, be correlated with the function of the site of modification.

We have shown that chemical modification of 30S subunits by photo-oxidation in the presence of Rose Bengal leads to loss of tRNA binding (6). In addition to the finding that modification of a small number of histidine residues in the ribosomal protein fraction accounts for this loss of activity, it was noticed that there was a parallel, but less pronounced, loss of tRNA binding due to modification of the 16S RNA, presumably from photo-oxidation of guanine residues. In order to examine more carefully the possibility that guanine modification leads to loss of function in the 30S subunit, we have used the guanine-specific reagent kethoxal (**I**) (7),



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which enables us to follow the effect of RNA modification without affecting the ribosomal proteins, and provides the possibility of attaching a covalently bound label to the site of reaction (8, 9). The experiments described here show that the reaction of 30S ribosomal subunits with kethoxal results in specific loss of tRNA binding, that the sites of reaction are protected from kethoxal by bound tRNA, and that the loss of function is correlated with the modification of 6 or 7 guanine residues in 16S RNA.

MATERIALS AND METHODS

Buffers. Buffer A: 10 mM $MgCl_2$–30 mM NH_4Cl–10 mM Tris·HCl (pH 7.8)–6 mM 2-mercaptoethanol. Buffer B: same as buffer A, but $MgCl_2$ concentration is 0.5 mM. Kethoxal reaction buffer: 0.1 M sodium cacodylate (pH 7.0)–10 mM $MgCl_2$. Buffer C: 30 mM Tris·HCl (pH 7.4)–20 mM $MgCl_2$–1 M KCl–6 mM 2-mercaptoethanol. Buffer D: same as buffer C, but 47.5 mM KCl.

Preparation of Ribosomes and Subunits. Frozen cells of *Escherichia coli* strain Q13 were obtained from General Biochemicals. Cells were suspended in 1.5 volumes of buffer A, and passed twice through an Aminco French pressure cell in 40-ml batches at 18,000 lb/in^2. Ribosomes were prepared from the extract by the method of Staehelin *et al.* (10). Ribosomal subunits were prepared by dialysis of 70S ribosomes against buffer B, followed by zonal centrifugation in a Spinco L3-40 centrifuge in a Ti-15 zonal rotor (11).

Kethoxal Reaction. Ribosomal subunits were suspended in kethoxal reaction buffer at a concentration of 6.0 mg/ml. (An extinction of 15.0 A_{260} was assumed equivalent to 1.0 mg of ribosomes or ribosomal subunits per ml.) 0.1 Volume of an aqueous solution of kethoxal (37 mg/ml) (Nutritional Biochemicals Corp., control no. 6322) was added to the ribosome suspension, and the reaction mixture was incubated at 37°. Aliquots were removed at specified time intervals, diluted with cold buffer A to a final volume of 1.0 ml, and precipitated at 0° by the addition of 0.65 ml of absolute ethanol (10). The precipitate was centrifuged at 10,000 rpm for 10 min in a Sorvall SS-34 rotor, and suspended in buffer A. Excess kethoxal was efficiently removed by this procedure. In protection experiments, 0.3 mg of poly(U) (Calbiochem) and 0.3 mg of *E. coli* B tRNA (Calbiochem) were added per mg of 30S subunits, and the mixture was incubated at 25° for 10 min before the addition of kethoxal.

Reaction with [^3H]Kethoxal. [*ethyl*-^{3}H]Kethoxal (5.2 Ci/mol, Schwarz–Mann) was diluted with unlabeled kethoxal to a final specific activity of 1.75 Ci/mol before use. 0.1 Volume of [^{3}H]kethoxal (37 mg/ml) was added to 430 μg of 30S subunits in 100 μl of kethoxal reaction buffer and incubated at 37°. At specific times, 10-μl aliquots were withdrawn and diluted with 3 ml of cold buffer B, washed through a Millipore HAWP-25 filter, and washed with three 3-ml portions of buffer B to remove unreacted kethoxal. Filters were dried and counted in 10 ml of 0.84% butyl PBD (Beckman) in a Beckman CPM 100 liquid scintillation counter. The efficiency of

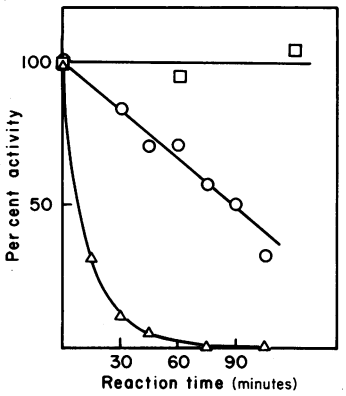


FIG. 1. Inactivation of 30S ribosomal subunits by kethoxal. 6 mg of subunits per ml in 0.1 M sodium cacodylate (pH 7.0)–10 mM $MgCl_2$ were mixed with 0.1 volume of an aqueous solution of kethoxal (37 mg/ml) and incubated at 37°. Aliquots were removed at the indicated times, and the reaction was terminated by chilling and ethanol precipitation of the 30S subunits. Subunits were suspended in buffer A and 30-μg aliquots were assayed for *in vitro* synthesis of polyphenylalanine in the presence of 60 μg of control 50S subunits, as described in *Methods*. (△), 30S subunits treated with kethoxal; (○), 30S subunits treated with kethoxal in the presence of 300 μg of poly(U) and 300 μg of tRNA per mg of subunits; (□), 30S subunits subjected to identical conditions as kethoxal-treated subunits, except for the omission of kethoxal. 100% activity corresponds to 92,600 cpm.

counting in this manner was determined by combustion analysis, performed by New England Nuclear.

Poly(U)-Directed Polyphenylalanine Synthesis. The procedure used was that previously described (6), except that the specific activity of [^{14}C]phenylalanine was 460 Ci/mol (Schwarz–Mann), and no prior incubation was performed before the addition of poly(U). Each assay tube contained 30 μg of 30S subunits and 60 μg of 50S subunits.

Poly(U)-Directed Binding of [^{14}C]Phenylalanyl tRNA. This was performed according to the method of Nirenberg and Leder (12), by the detailed procedure described (6).

Binding of [^{3}H]Poly(U). This was determined essentially by the filter-binding assay of Moore (13). The method was modified by the substitution of formaldehyde-treated 70S ribosomes for bovine serum albumin (6). The assay was linear within the range of ribosome quantities used.

Reconstitution of 30S Subunits. Total 30S ribosomal protein and 16S RNA were prepared from 30S subunits that had been reacted with kethoxal for 60 min as described above, or from control subunits that had been exposed to the same conditions, but with the omission of kethoxal (14). Reconstitution was by a modification (G. Thomas, unpublished) of the method of Traub and Nomura (15, 16). Total protein was dialyzed against buffer C and made up to a concentration of 0.8 mg/ml as determined by Folin assay (modification of Eggstein and Kreutz, as described by Bailey, ref. 17). 16S RNA was dialyzed against buffer D and diluted to a concentration of 10 A_{260} units/ml. RNA (45 μl) is first added to a glass conical centrifuge tube at 40°. After 5 min, 15 μl of protein solution are added. The reconstitution mixture is vigorously agitated at 40° for 40 min, and removed to an ice bath. Poly(U)-directed incorporation of phenylalanine is performed directly in the same tube.

Sedimentation Analysis of Ribosomal Subunits. Aliquots (30 μg) of 30S subunits in 100 μl of buffer B were layered on 3.6-ml linear sucrose gradients (5–20%) in buffer B and centrifuged at 59,000 rpm (400,000 × *g*) in an SB-405 rotor of an IEC B-60 ultracentrifuge for 1.2 hr at 5°. Gradients were analyzed by means of an ISCO gradient analyzer, and the absorbancy at 254 nm was recorded continuously. A parallel gradient containing untreated 30S subunits was run as a marker. The positions of duplicate markers were estimated to be reproducible to within 0.5 S.

RESULTS

Inactivation of 30S ribosomal subunits by kethoxal

The reaction of 30S subunits with kethoxal is accompanied by a rapid loss of *in vitro* protein synthesis, as measured by the poly(U)–phenylalanine system (Fig. 1). Subunits are protected from inactivation by poly(U) and tRNA, as observed previously in the case of Rose Bengal photo-oxidation (6) and tetranitromethane modification (18). Control subunits incubated under identical conditions, but in the absence of kethoxal, retain full activity. The results shown in Fig. 2 indicate that inactivation of protein synthesis is due to loss of tRNA binding. In contrast, particles inactive in protein synthesis are still able to bind synthetic messenger RNA (Fig. 2). Sedimentation analysis of particles inactivated by kethoxal shows no measurable change in sedimentation constant compared to untreated 30S subunits, but at later times there is an accumulation of faster sedimenting particles, which we interpret to be dimers (Fig. 3). Dimer formation is not correlated with loss of tRNA binding activity, and is not decreased by the presence of poly(U) and tRNA in the reaction mixture (Fig. 3*B*).

Identification of the site of functional modification

Total 30S protein and 16S RNA were extracted from 30S subunits inactivated by kethoxal and from control 30S subunits.

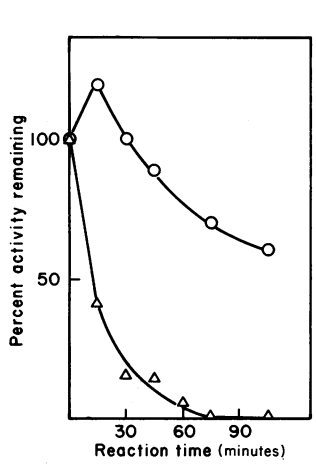


FIG. 2. Binding of poly(U) and tRNA to 30S subunits treated with Kethoxal. Aliquots of 30S subunits treated with kethoxal (30 μg) were assayed for (○) binding of [^{3}H]poly(U) by a filter-binding technique (13, 6) and for (△) poly(U)-directed binding of [^{14}C]phenylalanyl tRNA (12). The 30S subunits were those reacted with kethoxal in the experiment shown in Fig. 1. 100% activity in the poly(U)-binding assay was 3850 cpm, and in the tRNA binding assay was 1640 cpm.

These components were then reconstituted to give 30S particles having the four possible combinations of protein and RNA, and assayed for *in vitro* protein synthesis. It is evident from the results (Table 1) that the site of modification is the 16S RNA. The kethoxal–guanine adduct is somewhat labile at the high temperature (40°) and slightly basic (pH 7.4) conditions of ribosomal reconstitution (7, 19), thus giving rise to a partial reactivation of the 16S RNA. RNA modified by kethoxal retains its sedimentation contant of 16 S, with no evidence of dimer formation (Noller, unpublished).

Stoichiometry of kethoxal reaction with 30S subunits

The kethoxal reaction was performed under conditions identical to those of Fig. 1, but with [^{3}H]kethoxal. Aliquots were removed at various times, and the amount of kethoxal covalently bound to the 30S subunit was determined by a Millipore-binding technique (Fig. 4, see *Methods*). The absolute amount of label bound per ribosome was determined by combustion analysis of the kethoxal–ribosome complex bound to the filter after scintillation counting. Thus, about 10 mol of [^{3}H]kethoxal are bound per mol of 30S subunit after 50 min of incubation. In the presence of bound tRNA, however, only 3–4 mol of [^{3}H]kethoxal are bound, indicating that 6–7 mol of kethoxal react at sites protected by tRNA.

DISCUSSION

Although most of the functional assignments that have been made with the molecular components of ribosomes have dealt with the ribosomal proteins, some recent reports suggest that ribosomal RNA may be involved in ribosome function. Senior and Holland (20) and Bowman *et al.* (21) have reported the inactivation of 16S RNA promoted by colicin E3, and have shown that it is due to the scission of the RNA chain about 50 nucleotides from the 3′ terminus. There is evidence that resistance to kasugamycin (22, 23) and to erythromycin

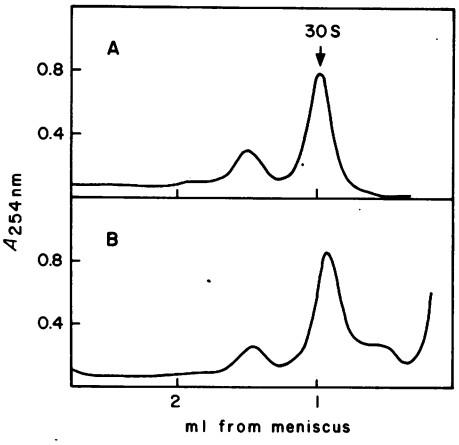


FIG. 3. Sedimentation of 30S subunits treated with kethoxal. 30S subunits treated with kethoxal as described in *Methods* for 75 min at 37° were applied to a 3.5-ml 5–20% sucrose gradient in buffer B and centrifuged for 1.2 hr at 59,000 rpm (400,000 × *g*) in an SB-405 rotor of an IEC B-60 ultracentrifuge. Gradients were pumped out and monitored continuously by means of an ISCO ultraviolet analyzer. (*A*) 30S subunits reacted for 75 min with kethoxal; (*B*) 30S subunits reacted for 75 min with kethoxal in the presence of tRNA and poly(U) (300 μg/mg of subunits). Sedimentation is from right to left. The 30S position was determined by comparison with untreated 30S subunits run on a parallel gradient.

TABLE 1. *Reconstitution of 30S ribosomal subunits from 16S RNA and total 30S proteins*

16S RNA	Total protein	Incorporation of [^{14}C]phenylalanine (cpm)	% activity
Control	Control	29,780	100
Control	Kethoxal	30,150	101
Kethoxal	Control	6,950	23
Kethoxal	Kethoxal	5,270	18

30S ribosomal subunits were reacted with kethoxal for 60 min at 37° as described in *Methods*. Control subunits were subjected to the same procedure, except for the omission of kethoxal. After removal of kethoxal by ethanol precipitation of 30S subunits, 16S RNA and total 30S protein were prepared, and the various particles were reconstituted and assayed as described in *Methods*. Each reconstitution experiment was performed in duplicate.

(J. Dahlberg, personal communication) can be caused by alterations in the normal pattern of methylation of 16S or 23S RNA. The colicin and kasugamycin studies suggest that the 3′ end of 16S RNA may play a role in protein synthesis. We have attempted to examine the possible functional involvement of 16S RNA by chemical modification of 30S ribosomal subunits with the guanine-specific RNA reagent kethoxal.

The results presented here show that kethoxal reacts with 30S ribosomal subunits to cause loss of messenger-directed tRNA binding. This is apparently due to modification of the tRNA binding site, since (*a*) there is only slight inactivation of the binding of poly(U) under conditions where tRNA binding has completely disappeared (Fig. 2), and (*b*) the site of the reaction leading to functional modification is strongly protected from kethoxal by the presence of tRNA (Figs. 1 and 4). The modification does not lead to a gross change in the conformation of the particle, since it retains its sedimentation coefficient of 30S. There is a gradual formation of a faster-sedimenting particle, which is probably a dimer of the 30S subunit, but this dimerization appears to be unrelated to the loss of tRNA binding.

Reconstitution experiments show that the site of functional alteration is within the 16S RNA molecule (Table 1). Previous

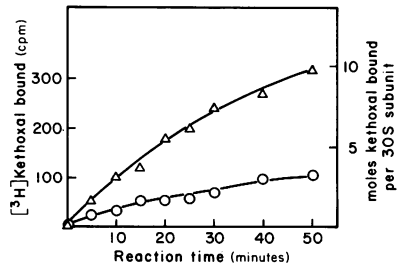
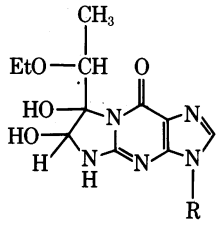


FIG. 4. Reaction of [^{3}H]kethoxal with 30S ribosomal subunits. The reaction was as described in *Methods*. (△), uptake of [^{3}H]-kethoxal by 30S subunits; (○), uptake of [^{3}H]kethoxal by 30S subunits in the presence of poly(U) and tRNA (300 μg/mg of subunits). Aliquots containing 60 μg of 30S subunits were taken at each point. The stoichiometry of the reaction was calculated after determination of counting efficiency by combustion analysis.

work has shown that chemical modification of ribosomal proteins in the 30S subunit by tetranitromethane (18) or Rose Bengal photo-oxidation (6) leads to specific loss of tRNA binding. Recently, we have identified a subset of the 30S ribosomal proteins that by functional and topological criteria appear to be part of the binding site for tRNA in the 30S subunit (Thomas, Rummel, and Noller, manuscript in preparation). The present results indicate that a portion of the 16S RNA may also be involved in the binding of tRNA. The observation that only a small number of guanine residues react with kethoxal under the conditions required for inactivation (Fig. 4) suggests that there are very few guanine residues in the 30S subunit that are both unpaired and accessible to the solvent (8, 9). Protection experiments (Figs. 1 and 4) indicate that about 6 out of 10 of these reactive guanines are in a region of the 30S subunit covered by bound tRNA. The actual number of residues involved may be somewhat more than 10 if there are partly reacted guanine residues included in the observed total. The small number of reactive guanine residues observed here is consistent with the relatively large size of the polynucleotide fragments obtained after digestion of intact 30S subunits with T1 ribonuclease, an enzyme that cleaves specifically at unpaired guanylic acid sites (P. Fellner, personal communication).

Kethoxal reacts with guanine to give a covalent adduct, most probably having the structure **II** (7, 19). Such a derivative



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would be expected to have lost all of the normal hydrogen-bonding capabilities of guanine. In addition, the presence of the bulky kethoxal moiety could interfere with the proper fit of a tRNA molecule to the ribosomal site. A possible functional role for guanine in tRNA binding was suggested by previous experiments from this laboratory (6). Rose Bengal photo-oxidation, which is known to be somewhat specific for guanine among the nucleoside bases (24), caused inactivation of 30S ribosomal subunits, and 16S RNA isolated from inactive particles was partially inactivated when assayed after reconstitution with control total protein. This observation has been confirmed by the present studies. Use of labeled kethoxal presents the possibility of identifying the location of the modified guanine nucleotides in the 16S RNA sequence.

In addition to providing identification of regions of the RNA that are possibly involved in ribosomal function, location of the sites protected by tRNA should provide information about the three-dimensional arrangement of the 16S RNA. Whether the structural modifications of ribosomal RNA reported here and elsewhere that correlate with alterations in ribosomal function reflect the existence of 'active sites' that are made up of portions of the ribosomal RNA, or whether they merely give rise to local changes in conformation that result in alteration of activity remains to be seen. If the former interpretation is correct, it follows that the tRNA binding site of the 30S ribosomal subunit is a composite structure, which includes portions of the 16S RNA, as well as several of the ribosomal proteins. Preliminary studies on ribosomal peptidyl transferase in this laboratory (Atchison, Moore, Moran, Hogan, and Noller, unpublished data) suggest that such composite structures may be a general feature of ribosome structure.

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