Ribonucleic Acid–Protein Cross-Linking within the Intact *Escherichia coli* Ribosome, Utilizing Ethylene Glycol Bis[3-(2-ketobutyraldehyde) ether], a Reversible, Bifunctional Reagent: Identification of 30S Proteins†

Linda A. Brewer* and Harry F. Noller

ABSTRACT: To obtain detailed topographical information concerning the spatial arrangement of the multitude of ribosomal proteins with respect to specific sequences in the three RNA chains of intact ribosomes, a reagent capable of covalently and reversibly joining RNA to protein has been synthesized [Brewer, L. A., Goelz, S., & Noller, H. F. (1983) *Biochemistry* (preceding paper in this issue)]. This compound, ethylene glycol bis[3-(2-ketobutyraldehyde) ether] which we term "bikethoxal", possesses two reactive ends similar to kethoxal. Accordingly, it reacts selectively with guanine in single-stranded regions of nucleic acid and with arginine in protein. The cross-linking is reversible in that the arginine– and guanine–bikethoxal linkage can be disrupted by treatment with mild base, allowing identification of the linked RNA and protein components by standard techniques. Further, since the sites of kethoxal modification within the RNA sequences of intact subunits are known, the task of identifying the components of individual ribonucleoprotein complexes should be considerably simplified. About 15% of the ribosomal protein was covalently cross-linked to 16S RNA by bikethoxal under our standard reaction conditions, as monitored by comigration of ^{35}S-labeled protein with RNA on Sepharose 4B in urea. Cross-linked 30S proteins were subsequently removed from 16S RNA by treatment with T_1 ribonuclease and/or mild base cleavage of the reagent and were identified by two-dimensional polyacrylamide gel electrophoresis. The major 30S proteins found in cross-linked complexes are S4, S5, S6, S7, S8, S9 (S11), S16, and S18. The minor ones are S2, S3, S12, S13, S14, S15, and S17.

Analysis of quaternary structure in the ribosome is essential to the complete understanding of the mechanism of protein synthesis. A major aspect of such an analysis requires the development of methods for obtaining information about the proximities of individual ribosomal proteins to specific sites in ribosomal RNA.[1] Although innovative techniques have been developed which have led to the localization of many proteins within the structure of the bacterial ribosome (Lake, 1980; Moore, 1980; Traut et al., 1980), an equivalent level of information and agreement concerning RNA conformation and RNA–protein interactions within the intact ribosome has been more difficult to obtain. Nevertheless, since the time when it was first suggested that most, if not all, of the ribosomal proteins would be found in domains containing RNA as well as protein (Kurland, 1974), new approaches have been developed for analyzing the physical proximity of specific ribosomal proteins to specific sequences in ribosomal RNA. Among those approaches which have yielded valuable results, the two which have proven most productive are the isolation and analysis of ribonucleoprotein fragments after ribonuclease digestion of ribosomes and the use of ultraviolet irradiation and/or chemical cross-linking reagents to introduce covalent bonds between ribosomal RNAs and proteins within the intact particle [see Zimmerman (1980) for a review].

For a number of reasons, more attention has recently been focused on developing covalent RNA–protein cross-linking reagents for these topographical studies. It was found that, upon unfolding of ribosomal subunits to release the smaller ribonucleoprotein fragments, proteins lose their specific binding sites on the RNA (Newton et al., 1975). While simple ultraviolet irradiation of ribosomes produces covalent cross-links, irradiation levels which produce more than 10% cross-linking of protein to ribosomal RNA cause cooperative unfolding of the subunits (Möller & Brimacombe, 1975; Gorelic, 1976). At lower levels of irradiation only three specific proteins were found to be cross-linked to RNA (Möller & Brimacombe, 1975; Baca & Bodley, 1976; Möller et al., 1978; Maly et al., 1980). Thus, other methods of cross-linking are needed, for this approach to be generally useful.

Until recently, bifunctional cross-linking compounds had not been extensively employed, largely due to the paucity of suitable reagents (Ulmer et al., 1978; Zimmerman, 1980). A number of RNA–protein reagents with varying length and chemical specificity have now been developed, and it has been possible to unambiguously identify the peptide and oligonucleotide components of cross-linked products (Wower et al., 1981). The approach has also been extended to the identification of proteins cross-linked to the RNA of the heterologous ribosomal subunit and therefore considered to be at or near the ribosomal subunit interface (Sköld, 1981). We have developed a bifunctional reagent, ethylene glycol bis[3-(2-ketobutyraldehyde) ether] which we term "bikethoxal", that has some advantages for investigating nucleic acid protein topography in a complex macromolecule such as the ribosome. Like other dicarbonyl compounds bikethoxal should react specifically with the guanidino function of arginyl (and possibly lysyl) residues in protein (Takahashi, 1968; Glass & Pelzig, 1978; Brewer et al., 1983) and with guanosyl residues (Staehelin, 1959; Shapiro & Hachmann, 1966; Litt & Hancock, 1967; Litt, 1969; Noller, 1974) at sites in RNA which

† From the Department of Biological Sciences, Stanford University, Stanford, California 94305 (L.A.B.), and Thimann Laboratories, University of California, Santa Cruz, California 95064 (H.F.N.). *Received January 31, 1983; revised manuscript received May 16, 1983.* This work was supported in part by Research Grants GM 17129 and RR 07135 from the U.S. Public Health Service to H.F.N. L.A.B was supported by A.A.U.W. and U.C. Fellowships.

* Address correspondence to this author at the Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305.

[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid disodium salt; RNA, ribonucleic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; RNase T_1, ribonuclease T_1 (EC 3.1.27.3); Gdn·HCl, guanidine hydrochloride.

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are not involved in base pairing interactions (Litt & Hancock, 1967; Litt, 1969; Noller, 1974). This high degree of specificity should minimize the effect of cross-linking on ribosome conformation. Furthermore, since bikethoxal is quite soluble in aqueous solution, this eliminates the uncertainty of the effect on ribosome structure of including organic solvents added to ribosomes with many bifunctional reagents. For example, various organic solvents have been included in reaction mixtures utilizing reagents such as *s*-triazine trichloride (Oste & Brimacombe, 1979), ethyl [(4-azidobenzoyl)amino]acetimide (Millon et al., 1980), (4-azidophenyl)glyoxal (Politz et al., 1981), 2-iminothiolane (Wower et al., 1981), esters of 3-[(2-bromo-3-oxobutyl)sulfonyl]propionic acid (Fink & Brimacombe, 1975; Fink et al., 1980), most of the azidoaryl imido esters (Rinke et al., 1980), and *N*-acetyl-*N'*-(*p*-glyoxylylbenzoyl)cystamine (Expert-Bezançon & Hayes, 1980)].

Since the sequences of most of the sites of kethoxal (a monofunctional dicarbonyl reagent of similar chemical specificity) modification are known (Noller, 1974; Chapman & Noller, 1977; Herr & Noller, 1978; Hogan & Noller, 1978), the analysis of the sites of protein cross-linking within the RNA chain will be greatly facilitated. Further simplifying the identification of cross-linked products is the lability of the covalent bond formed by the reagent in mildly basic solutions in the absence of borate ion.

We describe here the application of bikethoxal [ethylene glycol bis[3-(2-ketobutyraldehyde) ether] to the problem of determining the physical proximity of specific ribosomal proteins to ribosomal RNA. Preliminary reports of this work have been presented (Brewer & Goelz, 1979; Brewer, 1980). This bifunctional reagent forms cross-links that are reversible by mild base treatment, thereby leaving the involved macromolecules identifiable by conventional methods. Proteins so cross-linked to 16S RNA in the 30S ribosomal subunit of *Escherichia coli* have been identified by two-dimensional polyacrylamide gel electrophoresis.

Experimental Procedures

Preparation of Ribosomal Subunits and Proteins. Radioactively labeled ribosomal subunits were prepared according to the method of Brewer et al. (1983).

Unlabeled ribosomal subunits were prepared in parallel with radioactive subunits, except when larger amounts were required for the preparation of total 30S protein. In that case, *E. coli* MRE600 cells were purchased from Grain Processing Co. and were opened by sequential passage through a French pressure cell (Aminco) at 15 000 psi. Unlabeled total 30S ribosomal protein was extracted from 30S subunits with lithium chloride and urea by the method of Spitnik-Elson (1964), dialyzed against 0.03 M Tris-HCl, pH 7.7, 1.0 M NH_4Cl, 0.02 M $MgCl_2$, and 0.006 M 2-mercaptoethanol, and stored at −80 °C.

Cross-Linking Reaction Conditions. For each reaction, ^{35}S-labeled 30S ribosomal subunits (1.0×10^5–3.4×10^7 cpm) and unlabeled 30S subunits (to total 4.0 A_{260} units) were incubated at 40 °C for 20 min and then precipitated by adding 0.66 volume ethanol (−20 °C). Subunits were resuspended and reacted with bikethoxal according to the method described by Brewer et al. (1983) for the sedimentation experiments. Reaction mixtures were incubated for 2 h at 37 °C in each of the two buffers.

The initial bikethoxal reaction buffer was chosen because this was optimal for the formation of a single adduct with arginine (Brewer et al., 1983) at a pH where the adduct with guanine was expected to be unstable (Litt & Hancock, 1967; Litt, 1969; Noller, 1974). The subsequent incubation buffer was very similar to that used to modify single-stranded guanine sites in RNA with kethoxal (Litt, 1969; Noller, 1974). Following the removal of excess reagent and in the absence of borate ion as a stabilization factor, a proportion of the arginine adducts and the guanine adducts were expected to be unstable in mildly basic solutions (Litt & Hancock, 1967; Litt, 1969; Noller, 1974; Glass & Pelzig, 1978).

As controls, ^{32}P-labeled 30S and ^{35}S-labeled 30S ribosomal subunits were treated in the same manner as in the cross-linking reactions, except that bikethoxal was omitted.

Isolation and Purification of 30S Ribosomal Protein Cross-Linked to 16S RNA. Lyophilized cross-linked or control 30S ribosomal subunits were dissociated and fractionated by urea–Sepharose 4B chromatography as described by Brewer et al. (1983). Fractions were analyzed for radioactivity. For the ^{35}S-labeled 30S subunits that had been reacted with bikethoxal, the peak, in the excluded volume (fractions 5–8), was pooled. [The 16S RNA was found in the same position in the elution profile (see Results).]

A total of 2.5 volumes of ethanol (−20 °C) containing 10% (v/v) 0.2 M potassium borate, pH 7.0, was added to the pooled fractions. Borate is included since it is known to stabilize dicarbonyl–guanidino adducts (Litt, 1969; Glass & Pelzig, 1978). After storage at −20 °C for 2 h and centrifugation, the pellet was resuspended in 0.2 mL of 0.02 M potassium cacodylate, pH 7.0, 0.2 M sodium acetate, and 0.02 M potassium borate. The ethanol precipitation was performed 3 times; however, before the final precipitation, the sample was divided into three portions. The pellets were dried by vacuum desiccation. Dried pellets were resuspended and incubated for 1 h at 37 °C in one of the following buffers: 0.01 M Tris-HCl, pH 7.0, 0.02 M potassium borate, and 1 mM EDTA (neutral buffer); 0.01 M Tris-HCl, pH 8.5 (mild base); neutral buffer containing 0.01 mg of RNase T_1 [Sankyo, 20:1 (w/w) RNA–RNase] (RNase T_1). The three samples were lyophilized, dissociated, and fractionated exactly as described above.

The peak (fractions 13–21) of ^{35}S label found in the included volume following treatment with mild base or RNase T_1 was pooled and analyzed for radioactivity. [This is also the position where untreated ^{35}S-labeled 30S protein dissociated from 16S RNA migrates in the elution profile (see Results).]

Identification of Cross-Linked Proteins. Unlabeled total 30S protein (1.1 mg) was added to the pooled samples, fractions 13–21. At this point, the samples which had been treated with either mild base or with RNase T_1 prior to chromatography contained approximately 3.0×10^4 cpm of ^{35}S. The pool from the sample that had been treated with neutral buffer had a background level of radioactivity.

Protein samples were made 1% with 2-mercaptoethanol, heated 30 min at 37 °C, then precipitated with 0.25 volume of 50% trichloroacetic acid at 0 °C, washed with acetone and then ether, and dried by vacuum desiccation. To remove the oligonucleotide fragments from protein, the sample that had been treated with RNase T_1 was heated for 1 h at 37 °C with 0.01 mL of 30% triethylamine–carbonate. Lyophilized protein samples were analyzed on the two-dimensional gel system of Kaltschmidt & Wittmann (1970) as modified by Howard & Traut (1973) and by Thomas et al. (1975). Gels were both stained and autoradiographed (Sommer & Traut, 1976).

Results

The cross-linking reagent used in this study, ethylene glycol bis[3-(2-ketobutyraldehyde) ether], has been termed "bikethoxal" since it possesses two reactive ends similar to kethoxal (Brewer et al., 1983). The strategy used to identify the 30S

ribosomal proteins bound to 16S RNA by bikethoxal was to (1) unfold the cross-linked ^{35}S-labeled ribosome; (2) separate cross-linked from free 30S protein by Sepharose 4B chromatography; (3) release cross-linked protein from 16S RNA by either mild base or by digestion of the RNA with T_1 ribonuclease; (4) separate released proteins from material comigrating with 16S RNA by a second chromatographic step on Sepharose 4B; (5) remove the remaining oligonucleotide fragments from nuclease-released cross-linked proteins by base treatment prior to electrophoresis; (6) identify proteins which had been cross-linked to 16S RNA by subjecting separated proteins to electrophoresis on two-dimensional polyacrylamide gels. Positions of the ^{35}S-labeled, cross-linked proteins were determined by autoradiography and were matched to the exact position of stained, control proteins run on the same gel.

Isolation of Cross-Linked Proteins. When ^{35}S-labeled 30S ribosomal subunits were treated with bikethoxal, about 15% of the protein was cross-linked to 16S RNA under these incubation conditions (Figure 1B). Cross-linking was measured as the proportion of ^{35}S label which comigrated with 16S RNA during Sepharose 4B chromatography (in urea and high salt), after unfolding of the subunit by incubation with Gdn·HCl. Such chromatography effected a rapid and complete separation of 30S ribosomal protein from the peak of 16S RNA in untreated subunits, as shown in Figure 1A.

In order to confirm that the ^{35}S-labeled protein comigrating with 16S RNA was in fact cross-linked and in order to separate cross-linked proteins from 16S RNA, the peak (fractions 5–8) of the RNA plus RNA–protein complexes was collected by ethanol precipitation. The putative 16S RNA–30S protein cross-links were tested in three separate ways: (1) a control incubation, (2) a mild base treatment to reverse the cross-linking reaction, and (3) a T_1 ribonuclease digestion to remove most of the RNA from the protein. The stability of the RNA–protein cross-link was confirmed by the fact that pooled RNA–protein complexes incubated in a control buffer (containing borate ion at neutral pH) continued to chromatograph with 16S RNA on urea–Sepharose 4B columns [Figure 1C-(1)]. Covalent attachment of the ^{35}S label to RNA was also confirmed since a major portion of the cross-linked protein found in RNA–protein complexes chromatographed in the same position as free protein (Figure 1A) following incubation in mild base (pH 8.5) in the absence of borate and in the absence of excess reagent (to reverse the cross-linking process) or with T_1 ribonuclease (to release attached proteins from the 16S RNA peak). Similarly, after nuclease digestion a portion of the cross-linked protein chromatographed in the same position as free protein (Figure 1A) as can be seen in Figure 1C(3). T_1 oligonucleotides also chromatograph in this position (data not shown). Under stronger nuclease digestion conditions virtually all of the ^{35}S-labeled protein comigrating with 16S RNA was released (data not shown); however, the additional nuclease severely overloaded the sample gel of subsequent two-dimensional polyacrylamide gels.

Identification of Cross-Linked 30S Proteins. ^{35}S-Labeled ribosomal proteins cross-linked to 16S RNA were identified by autoradiography of stained two-dimensional polyacrylamide gels (Kaltschmidt & Wittmann, 1970). Untreated, unlabeled 30S protein was added to radioactive, cross-linked protein prior to electrophoresis to provide a stainable reference for identification of cross-linked 30S proteins in the autoradiogram. Also, ^{35}S-labeled protein released from 16S RNA by treatment with T_1 RNase was incubated in mild base to remove covalently bound T_1 oligonucleotides facilitating identification of the cross-linked proteins.

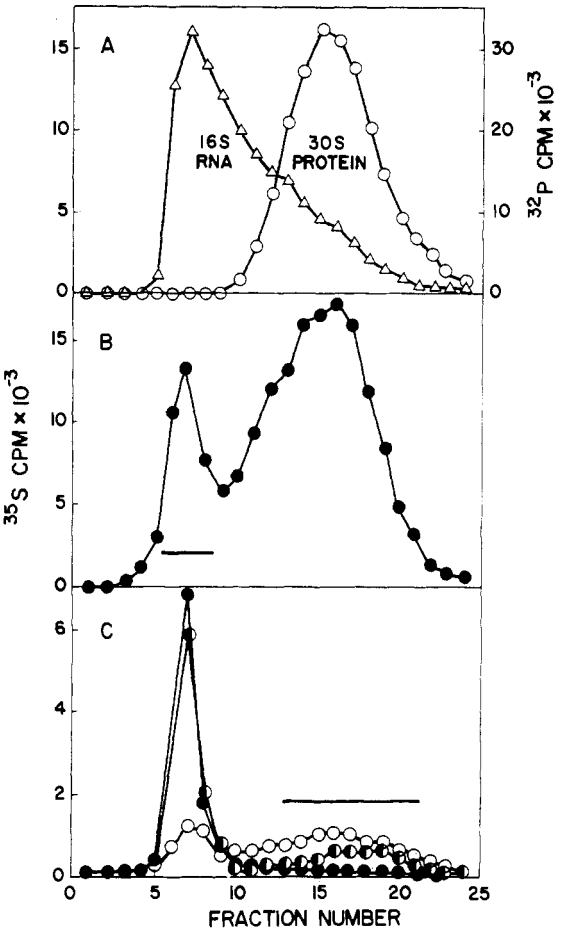

FIGURE 1: Separation of 30S protein cross-linked to 16S RNA from non-cross-linked 30S protein. Radioactively labeled 30S ribosomal subunits were dissociated in Gdn·HCl and fractionated on a column of Sepharose 4B in 8 M urea, 0.5 M sodium acetate, 20 mM potassium borate, and 20 mM potassium cacodylate (pH 7.0) at 4 °C as described under Experimental Procedures. (A) ^{32}P-Labeled 16S RNA (△) or ^{35}S-labeled 30S protein (○) in 30S ribosomal subunits is incubated in the absence of bikethoxal (control). Trailing of the RNA peak is attributable to some radiolytic cleavage incurred on storage. (B) ^{35}S-Labeled 30S protein (●) in 30S ribosomal subunits treated with bikethoxal (cross-link). (C) ^{35}S-Labeled 30S protein cross-linked to 16S RNA (obtained from fractions 5–8 pooled from B) were divided into three portions and treated as follows, as described in detail under Experimental Procedures: (1) heated at pH 7.0 in the presence of borate (●) (stabilized RNA–protein cross-link); (2) heated at pH 8.5 in the absence of borate (○) (cross-link reversed by mild base); (3) heated at pH 7.0 in the presence of borate and T_1 ribonuclease (◐) (nuclease digestion of 16S RNA in the RNA–protein cross-link). Fractions 13–21 were pooled for identification of 30S protein cross-linked to 16S RNA.

The 30S proteins cross-linked to 16S RNA and released by mild base are shown in the autoradiogram in Figure 2B. A tracing of the stained gel used to identify the spots on the autoradiogram is also shown (Figure 2A). The cross-linked proteins (which comigrated in exactly the same positions as untreated 30S protein) were identified. The major ones were S4, S5, S6, S7, S8, S9 (S11), S16, and S18. The minor ones were S2, S3, S12, S13, S14, S15, and S17.

The anomalous spot appearing near S1 on the stained gel and in the autoradiogram was not identified; however, it appears to have a similar mobility to a stained (but unidentified) region in the original Kaltschmidt & Wittmann (1970) gel system. They report that proteins found in this portion of the gel vary with the way in which the ribosomes and their proteins are prepared. It is unlikely to be an oxidation product of any of the identified 30S ribosomal proteins, since it would be

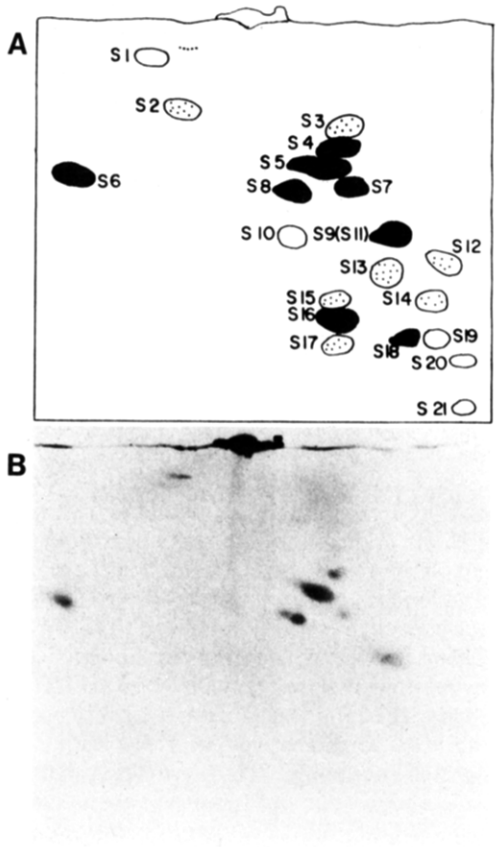

FIGURE 2: Identification of 30S proteins cross-linked to 16S RNA by reaction with bikethoxal and removed by mild base. (A) Two-dimensional gel electrophoresis of total 30S subunit protein. Tracings of the stained proteins are filled to indicate the position of the major proteins found in cross-linked complexes or stippled to indicate the minor ones, as shown in the autoradiogram in (B); the stippled line indicates an anomalous stained region, also found in the corresponding autoradiogram (see text). (B) Autoradiogram of ^{35}S-labeled cross-linked 30S subunit proteins. Cross-linked proteins were released by base treatment and were obtained by pooling fractions 13–21 [Figure 1C(2)].

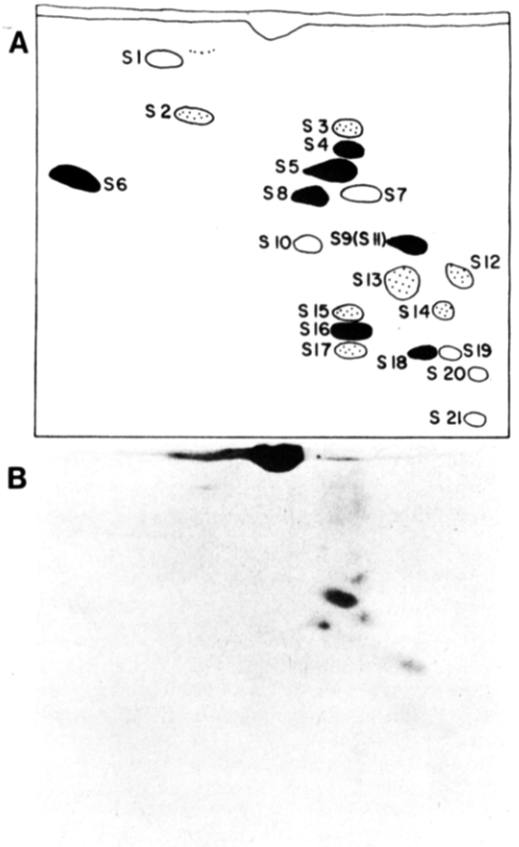

FIGURE 3: Identification of 30S protein cross-linked to 16S RNA by reaction with bikethoxal and digested with T_1 ribonuclease. (A) Two-dimensional gel electrophoresis of total 30S subunit protein. Tracings of the stained proteins are as in Figure 2A. (B) Autoradiogram of ^{35}S-labeled cross-linked 30S subunit proteins. The 16S RNA–30S protein cross-linked complexes were treated with T_1 ribonuclease. Ribonucleoprotein complexes were isolated, as shown in Figure 1, lower elution profile [C(3)], and fractions 13–21 containing cross-linked ribonucleoproteins were pooled. Protein and RNA moieties were cleaved prior to electrophoresis by removing the cross-linker with mild base.

expected that oxidized proteins would become more acidic and shift toward the anode in both dimensions (Kaltschmidt & Wittmann, 1970).

Cross-linked proteins released from 16S RNA by ribonuclease digestion are shown in the autoradiogram in Figure 3B. Again, a tracing of the stained gel used to identify the spots on the autoradiogram is also shown (Figure 3A). The cross-linked proteins (which comigrated in exactly the same positions as untreated 30S protein) were identified. They were identical with those found after release of cross-linked protein from 16S RNA by reversal of the cross-linking reaction. This is the expected result since the number and identity of the proteins released from the RNA peak should be independent of the method of removal.

The pattern of appearance of cross-linked 30S protein was highly reproducible. Furthermore, no spots appeared on the gels if ^{35}S-labeled 30S subunits had not been treated with bikethoxal but had been treated similarly in all other respects or if protein had not been released from the RNA peak.

Discussion

The complexity and asymmetry of ribosomal structure require the development of novel techniques for its elucidation. Although the use of RNA–protein cross-linking as a probe of ribosomal topography is yet to be fully exploited, it is beginning to produce valuable information. This type of information is different from that gained from the binding of individual proteins to RNA. Binding studies give a measure of physical association in the in vitro assembling ribosome, whereas cross-linking studies give information about the proximity of RNA and protein within intact subunits. It has been pointed out that cross-linking studies offer the possibility of establishing topographical neighborhoods between protein and RNA, which in many cases might never be detected by binding studies (Ulmer et al., 1978).

Therefore, it is of interest to compare the results of the binding studies to results obtained by employing RNA–protein reagents. In Table I the proteins are listed which were observed to be in the neighborhood of RNA in 30S ribosomal subunits, with bikethoxal cross-linking as a probe of their proximity. Table I also shows the proteins which have been observed to bind individually to 16S RNA. Eight of the 10 RNA "binding proteins" [see Zimmerman (1980) for a review] appear to be cross-linked to RNA in this study with bikethoxal. Interestingly, two of these proteins, S2 and S5, have only more recently been shown to exhibit independent RNA binding properties, due to the necessity of using a more gentle method of extracting the proteins to retain the RNA binding function (Littlechild et al., 1977; Littlechild & Malcolm, 1978).

The general result that a majority of the ribosomal proteins can be cross-linked to ribosomal RNA is also suggested in a number of other studies with RNA–protein cross-linking reagents. The pattern of proteins observed to be cross-linked

Table I: Relative Extent of Cross-Linking to 16S RNA for the 30S Ribosomal Proteins by Bikethoxal[a]

30S proteins	binding	extent of cross-linking
S1		(–)
S2	(S)	±
S3		+
S4	(A, S)	+ + +
S5	(S)	+ + +
S6		+ + +
S7	(A, S)	+ + +
S8	(A, S)	+ + +
S9 (S11)		+ + +
S10		(–)
S12		+
S13	(A, S)	+
S14		+
S15	(A, S)	±
S16		+ +
S17	(A, S)	+
S18		+ +
S19	(S)	(–)
S20	(A, S)	(–)
S21		(–)

[a] The data were compiled from four independent experiments. The number of (+) symbols indicates increasing extent of cross-linking; the (–) symbol indicates no detectable cross-linking. RNA "binding proteins" [see Zimmerman (1980), for review] are indicated by the following: A, proteins extracted with acetic acid and urea that are generally thought to bind RNA which is extracted with phenol and SDS (Mizushima & Nomura, 1970; Schaup et al., 1970, 1971; Garrett et al., 1971; Muto et al., 1974; Held et al., 1974); S, proteins extracted with high salt (Littlechild et al., 1977; Littlechild & Malcolm, 1978).

differs somewhat in each case, but this would be expected considering the different reaction conditions and reagent specificities employed. All but two of the 30S proteins were cross-linked to RNA by formaldehyde (Möller et al., 1977). Although the reaction with formaldehyde, another RNA–protein reagent, is also reversible (Möller et al., 1977; Pon et al., 1977), it is too labile, creating variability in the proteins observed to be cross-linked and difficulty in the isolation of intact ribonucleoprotein complexes (Ulmer et al., 1978; Zimmerman, 1980). At least half of the ribosomal proteins were covalently bound to one or the other of the major ribosomal RNA species by diepoxybutane (Bäumert et al., 1978; Sköld, 1981). Also, in the case of the 30S subunit Rinke et al. (1980) found that almost all of the proteins were involved in the cross-linking reaction which employs the heterobifunctional RNA–protein reagent (*p*-azidophenyl)acetic imido ester. Further, a compound which reacts in a very similar fashion, 2-iminothiolane, yielded RNA cross-links with most of the ribosomal proteins (Wower et al., 1981).

More specifically, ribosomal protein S7, which has been cross-linked to U_{1239} of 16S RNA by UV irradiation (Möller et al., 1978; Zwieb & Brimacombe, 1979), was also cross-linked by bikethoxal. All of the proteins cross-linked to RNA with (4-azidophenyl)glyoxal (Politz et al., 1981), a compound with one reactive group similar to bikethoxal, are also cross-linked to RNA by bikethoxal. These proteins are S2, S3, S4, S5, S7, and S12. Also, all of the proteins cross-linked to 16S RNA in the 30S subunit by ethyl [(4-azidobenzoyl)amino]-acetimidate, a reagent with one reactive end that also modifies protein NH_2 groups, are also cross-linked by bikethoxal. These proteins are S3, S4, S5, S7, S9, S12, S13, S14, S16, S17, and S18. The major 30S proteins cross-linked to RNA with 2-iminothiolane (Wower et al., 1981), S3 and S4, are also cross-linked by utilizing bikethoxal. In a study employing a reagent, diepoxybutane (Sköld, 1981), with a different chemical specificity and a much shorter bridge length, the same number of proteins were cross-linked in the 30S subunit, and in a comparison of the 21 proteins in the 30S subunit, 16 were in agreement.

The length of the reagent is unlikely to account for the number of proteins found near RNA in the 30S subunit. Since the length of the covalent bridge formed by bikethoxal is expected to be on the order of 8–9 Å, the distances separating the cross-linked proteins and RNA within the ribosomal subunit are relatively small. This argument is supported by the finding that a shorter version of the compound, (*p*-azidophenyl)acetic imido ester (a reagent with an expected bridge length approximately the same as that of bikethoxal), does not lead to a more selective cross-linking reaction, but merely to a lower yield of the same products (Rinke et al., 1980). It should also be noted that all cross-linking reagents employed in a complex, dynamic structure like that of the ribosome will detect proteins proximal to RNA on a time scale compatible with the reaction kinetics of the reagent.

The possibility that some of the proteins appearing to be cross-linked to RNA were recovered as protein–protein–RNA complexes cannot be entirely ruled out until we have successfully identified the sequences of oligonucleotides attached to single specific ribosomal proteins obtained from isolated ribonucleoprotein complexes. However, this possibility is unlikely since the reaction conditions were chosen to minimize protein–protein cross-links. The observation that no higher molecular weight protein bands appeared between the RNA and control 30S protein when subunits were reacted with bikethoxal and separated by SDS–agarose gel electrophoresis (Brewer et al., 1983) argues against the formation of protein–protein complexes in significant amounts. Further, as we have shown here, releasing cross-linked proteins from 16S RNA by mild base reversal of the reaction or by limited T_1 RNase digestion gave the same pattern of proteins. That is, it would be expected that larger protein–protein–RNA complexes would be more protected from nuclease attack. Thus under the conditions of incomplete digestion used in this study, protected complexes would not be released from the peak of RNA and would therefore not appear in the pool of cross-linked proteins. However, no proteins are missing from the pattern in this case. Along the same lines, the pool of proteins released from cross-link with RNA was made in a manner that would exclude larger protein–protein complexes (^{35}S-labeled material migrating at the leading edge of the protein peak).

In conclusion, these initial studies indicate that bikethoxal is a promising bifunctional reagent for RNA–protein cross-linking in the *E. coli* ribosome. Since it is capable of covalently, but reversibly, joining a large proportion of the small subunit proteins to 16S RNA under mild conditions, it may prove a good general reagent. Bikethoxal is quite specific and can be used under very mild conditions. Thus, it would be expected to have minimal effect on ribosome conformation. Further, since the sites of kethoxal modification of *Escherichia coli* ribosomal RNAs are known (Noller, 1974; Chapman & Noller, 1977; Hogan & Noller, 1978; Herr & Noller, 1978), the task of identifying the components of individual ribonucleoprotein complexes should be considerably simplified. Furthermore, the fact that the cross-link induced by bikethoxal is stable allows the isolation of individual ribonucleoprotein complexes and that it is also reversible allows the characterization of specific oligonucleotides and peptides from adducts by conventional techniques.

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Registry No. Bikethoxal, 84031-85-6; RNase T_1, 9026-12-4.

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