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Reductive Alkylation of Ribosomes as a Probe to the Topography of Ribosomal Proteins*

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Escherichia coli ribosomes were treated with a number of different aldehydes of various sizes in the presence of $NaBH_4$. After incorporation of either ^3H or ^{14}C, the ribosomal proteins were separated by two-dimensional polyacrylamide-gel electrophoresis and the extent of alkylation of the lysine residues in each protein was measured. The same pattern of alkylation was observed with the four reagents used, namely formaldehyde, acetone, benzaldehyde and 3,4,5-trimethoxybenzaldehyde. Every protein in 30S and 50S subunits was modified, although there was considerable variation in the degree of alkylation of individual proteins. A topographical classification of ribosomal proteins is presented, based on the degree of exposure of lysine residues. The data indicate that every protein of the ribosome has at least one lysine residue exposed at or near the surface of the ribonucleoprotein complex.

The structural organization of the proteins and RNA within *Escherichia coli* ribosomes has received considerable attention in the last few years. In the absence of suitable crystals for X-ray analysis considerable reliance has been placed on solution chemistry to probe the three-dimensional structure of the ribosome. Until now, three approaches have been used to probe for exposed regions of proteins on the surface of the ribosome: (1) susceptibility to chemical modification (Craven & Gupta, 1970; Kahan & Kaltschmidt, 1972), (2) susceptibility to trypsin digestion (Craven & Gupta, 1970; Chang & Flaks, 1970; Spitnik-Elson & Breiman, 1971; Crichton & Wittmann, 1971), and (3) accessibility to protein-specific antibodies (Stöffler *et al.*, 1973).

The present paper describes the results of a study on the accessibility of the lysine residues of individual proteins by chemical modification of 30S and 50S subunits with a series of aldehydes and $NaBH_4$. In this procedure the unstable Schiff base formed between the ε-amino group of a lysine residue and an aldehyde or ketone was reduced with $NaBH_4$ to give the stable ε-*N*-alkyl-lysine derivative. Either radioactive aldehyde or radioactive $NaBH_4$ was used to monitor the reaction. A series of aldehydes of increasing size was used as a control against possible diffusion of the reagents into the 'interior' of the ribosome.

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Experimental

Materials

KB^3H_4 (306mCi/mmol), [^{3}H]formaldehyde (100mCi/mmol), [^{14}C]acetone (11mCi/mmol) and [^{14}C]benzaldehyde (1.82mCi/mmol) were purchased from Amersham–Buchler, Braunschweig, Germany. [^{14}C]Formaldehyde (10mCi/mmol) and [^{14}C]phenylalanine were obtained from New England Nuclear Corp., Dreieichenhain, Germany. TMA buffer was 10mM-Tris–HCl, 10mM-$MgCl_2$, 20mM-NH_4Cl and 6mM-β-mercaptoethanol, pH7.8. BMK buffer was 100mM-sodium borate, 10mM-$MgCl_2$, 20mM-KCl, 6mM-β-mercaptoethanol, pH8.5. All other reagents were high-purity preparations obtained from commercial sources.

Methods

Ribosome preparation. Ribosomes from *E. coli* strain A19 harvested in late exponential phase of growth were isolated by standard procedures (Traub *et al.*, 1971). From them 30S and 50S subunits were separated by sucrose-density-gradient centrifugation, isolated by ethanol precipitation and stored at −80°C in 5mM-magnesium acetate.

Reductive alkylation. Reductive alkylation was carried out as previously described (Moore & Crichton, 1973). Reductive isopropylation, benzylation and 3,4,5-trimethoxybenzylation were carried out as follows: to a solution of 20 E_{260} units of 30S or 50S ribosomes in 500μl of BMK buffer at 0°C, 80μl of a solution of benzaldehyde or 3,4,5-trimethoxybenzaldehyde (0.5mM in ethanol), or 100μl

Table 1. *Reductive alkylation of 30S and 50S ribosomal subunits with a series of aldehydes of increasing size*

Distribution of radioactivity is corrected for percentage of material remaining at the origin of the polyacrylamide plate (Weber, 1972). Each value is an average determined from four to nine polyacrylamide plates; standard errors vary from 10 to 30%. For other details see the text.

	30S subunit (% of total incorporation)					50S subunit (% of total incorporation)			
Protein	Form-aldehyde	Acetone	Benz-aldehyde	Trimethoxy-benzaldehyde	Protein	Form-aldehyde	Acetone	Benz-aldehyde	Trimethoxy-benzaldehyde
S1*	8	8	7.5	9	L1	11	10.5	8	9
S2	6	5.5	6	7	L2	1.5	4	3.5	3.5
S3	10	6	6	7	L3	5	5	3	4
S4	6	7	8	11	L4	4	3.5	3	4.5
S5	10	13	9	12	L5	4	3.5	3	3.5
S6	5	6.5	6	5	L6	3	2.5	3	3
S7	6	5	4	4.5	L7	6	6	8	6.5
S8	2	3	3.5	2.5	L8+L9	7	6	7	7
S9	7	7.5	8	8	L10	3	2	2	3
S10	8	6	8	7.5	L11	5	6	4	5.5
S11	3	3	3	2	L12	5	1.5	2	1.5
S12	1.5	0.5	1	0.5	L13	2.5	2.5	2	2.5
S13	4	7.5	7.5	6.5	L14	1	1.5	1.5	1
S14	2	1	2	1.5	L15	3	3	2	2
S15	3.5	4	4	3	L16	2	1.5	2	1.5
S16	1	1	1.5	1	L17	1.5	2	3	2
S17	1.5	1	1.5	1.5	L18	4	3.5	4.5	4
S18	1.5	1.5	2.5	2	L19	5	3.5	4.5	4
S19	5	4	4	3	L20	0.5	0.4	0.6	0.6
S20	4	6	6	5	L21	1.5	2	2	2.5
S21	3	3	4	3.5	L22	5	5	4.5	3.5
					L23	2	1.5	2	1.5
					L24	4	2.5	2	1.5
					L25	3.5	4.5	4.5	5
					L27	1.5	2	3	3
					L28	0.3	0.6	1	1
					L29	2	1.5	2	2.5
					L30	1.5	2.5	2	2
					L31	0.1	0.3	0.2	0.2
					L32	1.5	4	3	3
					L33	3	3	4	4
					L32	1.5	4	3	3
					L33	3	3	4	4

* Data for protein S1 were extremely variable. The values given represent averages.

of a solution of acetone (0.1 M in water) was added. After 10min, four 40μl (or 10μl when acetone was used) additions of an aqueous solution of KBH_4 (10mg/ml) were made at 2min intervals. The reaction mixture was left with occasional shaking at 0°C for 10min, and then dialysed for 24h against several changes of TMA buffer. Either the aldehyde or borohydride was radioactive. When KB^3H_4 was used, the number of alkyl groups incorporated into the sample was calculated on the assumption that one hydrogen atom derived from KBH_4 is incorporated per alkyl group per lysine residue (Means & Feeney, 1968).

Two-dimensional polyacrylamide-gel electrophoresis. Unlabelled ribosomes (40E_{260} units of 30S or 80E_{260} units of 50S) were added to each labelled sample, the RNA (which was not labelled) was removed by treatment with 67% (v/v) acetic acid and the proteins were separated by two-dimensional gel electrophoresis (Kaltschmidt & Wittmann, 1970). Immediately before electrophoresis a portion of the sample dissolved in unpolymerized sample gel was removed and counted for radioactivity in InstaGel (Packard). After electrophoresis spots on the plates were cut out, solubilized with H_2O_2 and the radioactivity was assayed (Kahan & Kaltschmidt, 1972). The radioactivity recorded for each protein was corrected for the fractionality (i.e. stoicheiometry) of each protein in ribosomes isolated from a rich medium

(Deusser, 1972) and for the percentage of material remaining at the origin of polyacrylamide plates (Weber, 1972). The position of the spots on the plates is unaffected by reductive alkylation.

Functional activity. Functional activities of 30S and 50S subunits in poly(U)-dependent polyphenylalanine synthesis were determined by the method of Traub *et al.* (1971).

Ultracentrifugation. Analytical ultracentrifugation runs were carried out in a Titanium-An-G five-place rotor at 36000rev./min and 6°C in a Beckman model E ultracentrifuge equipped with u.v. optics, multiplex accessory and photoelectric scanner.

Results

The product of reductive alkylation when formaldehyde is used is ε-*N*-dimethyl-lysine (Means & Feeney, 1968), whereas with acetone, benzaldehyde or 3,4,5-trimethoxybenzaldehyde monoalkylation occurs (Means & Feeney, 1968; G. Moore & N. L. Benoiton, unpublished work). In general about 50 alkyl groups were incorporated into 30S ribosomes when formaldehyde, benzaldehyde or 3,4,5-trimethoxybenzaldehyde were used. When acetone was used, 10–30 isopropyl groups were incorporated. Twice as many groups were incorporated into intact 50S subunits. There are 200 lysine residues per 30S particle and 400 lysine residues per 50S particle. Therefore 10–15% of the total lysine content of intact ribosomes was dimethylated or isopropylated, and 20–30% was benzylated or 3,4,5-trimethoxybenzylated.

The functional activities [poly(U) assay] and sedimentation profiles (ultracentrifugation) of subunits were unaffected by any of the modifications described. However, unlabelled reagents were used in the isopropylation, benzylation and 3,4,5-trimethoxybenzylation of subunits that were subsequently assayed for functional activity, and the extent of alkylation of the subunits in each of these cases was therefore not known.

The distribution of radioactivity among the proteins of 30S and 50S subunits in the four alkylation reactions is given in Table 1. Within the limits of experimental error, the same percentage of total incorporation was observed for each protein regardless of the aldehyde used. The distribution of radioactive label was also independent of the number of alkyl groups incorporated into the subunits; in experiments using low concentrations of [^{3}H]formaldehyde of high specific radioactivity, 30S subunits were trace-labelled and the distribution of the radioactive label remained the same.

In Table 2 the extent of methylation of the lysine residues of each protein has been computed. As a control, 30S and 50S ribosomal proteins isolated by treatment with 67% acetic acid and dissolved in BMK buffer, pH8.5, containing 4M-guanidinium hydrochloride were reductively methylated with formaldehyde and $NaBH_4$. By comparing for each protein the percentage methylation of the lysine residues in intact ribosomes and in dissociated ribosomal proteins, it is possible to assess the relative degree of exposure of the protein. This classification assumes that the lysine residues are distributed uniformly over the surface of the ribosome. Theoretically it should be possible to determine the degree of accessibility of individual proteins solely from the percentage methylation of the lysine residues in intact subunits. However, some of the factors involved in these calculations, such as determination of the fractionality and the amount remaining at the origin, may be erroneous and, as a control, a reference was obtained by modifying proteins in a completely unfolded state in guanidinium hydrochloride. Since the same factors are used in calculations with the reference as well as the probe, possible errors cancel out when the two are compared.

Discussion

From the data in Table 1 it is apparent that each of the four aldehydes used for reductive alkylation of intact ribosomes gives the same pattern of alkylation of the proteins, and it is likely that the same lysine residues react with all four reagents. Since it is unlikely that a molecule as large as 3,4,5-trimethoxybenzaldehyde could diffuse into the interior of the ribonucleoprotein complex and react with buried lysine residues, it must be assumed that even formaldehyde, which is a small molecule and potentially able to diffuse into the ribosome, reacts only with lysine residues on the surface of the complex.

The extent of alkylation of a lysine ε-amino group in intact ribosomes is dependent on its accessibility to the reagent and its pK_a in the micro-environment. Residues on the surface of the subunit which are in contact with the solvent will be alkylated more readily than internal residues or lysine residues having an abnormally high pK_a. The ε-amino groups of lysine residues which are involved in salt links are presumably sterically shielded to some extent. They also have effectively higher pK_a values, and at pH8.5 should react more slowly than lysine residues not involved in salt linkages. In agreement with this, the extent of methylation of ribosomes bound to a poly(U) template was 10–15% less than that of unbound ribosomes (G. Moore, unpublished work), presumably because some of the lysine residues are 'concealed' by salt linking to the phosphate groups on the messenger RNA. Further, examination of the data in Table 2 shows that the lysine residues of ribosomal RNA-binding proteins S4, S7, S8, S13, S15, S20, L2, L6, L16, L17, L19, L20, L23, L24, L17 and L25 (Mizushima & Nomura, 1970; Schaup *et al.*, 1970, 1971;

Table 2. *Reductive methylation of intact ribosomal subunits and mixtures of proteins derived from ribosomal subunits*

Distribution of radioactivity is corrected for percentage of material remaining at the origin of the polyacrylamide plate (Weber, 1972). Each value is an average determined from between four and nine polyacrylamide plates; standard errors vary from 10 to 30%. The percentage of total protein lysine residues that were methylated was calculated from experiments in which 44 and 96 methyl groups were incorporated into intact 30S and 50S subunits respectively, and 92 and 186 methyl groups incorporated into dissociated proteins derived from 30S and 50S subunits respectively. The extent of methylation was determined as dimethyl-lysine content after correction for rich medium fractionality (Deusser, 1972) and percentage of material remaining of the origin on the two-dimensional plate (Weber, 1972). Reductive methylation was carried out on mixtures of proteins derived from 30S or 50S subunits dissolved in BMK buffer, pH 8.5, containing 4 M-guanidinium hydrochloride. The degree of exposure of the lysines is indicated by the number of + signs; ++++ indicates the highest degree of exposure but does not indicate that all the lysines of a particular protein are exposed in the intact subunit.

	% of total incorporation		% of total protein lysine methylated		
Protein	Intact subunit	Dissociated proteins	Intact subunit	Dissociated proteins	Exposure classification
S1*	8	2.5	29	20	(++++)
S2	6	4.5	14	20	+++
S3	10	6	12	15	+++
S4	6	11.5	4	16	+
S5	10	12	12	24	++
S6	5	3	50	50	++++
S7	6	5	8	14	++
S8	2	5	4	19	+
S9	7	9	12	31	+
S10	8	4	50	50	++++
S11	3	2	6	8	+++
S12	1.5	2	11	29	+
S13	4	5	11	27	++
S14	2	3	11	31	+
S15	3.5	6	8	27	+
S16	1	2	4	15	+
S17	1.5	2	3	8	+
S18	1.5	2	10	30	+
S19	5	4.5	14	26	++
S20	4	4.5	10	22	++
S21	3	2.5	11	19	++
L1	11	11	30	52	++
L2	1.5	9	4	19	+
L3	5	4	14	33	++
L4	4	6	20	34	++
L5	4	3.5	10	16	++
L6	3	3	14	28	++
L7	6	3	26	23	++++
L8+L9	7	2.5	—	—	−
L10	3	5.5	18	30	++
L11	5	3	26	32	+++
L12	5	2.5	34	36	++++
L13	2.5	6	10	50	+
L14	1	2	10	41	+
L15	3	5.5	16	58	+
L16	2	4	12	46	+
L17	1.5	3	8	30	+
L18	4	3.5	14	24	++
L19	5	2.5	10	14	+++
L20†	0.5	1	—	—	+
L21	1.5	3.5	10	44	+
L22	5	5.5	16	35	++
L23	2	3	8	25	+
L24	4	4.5	10	24	++
L25	3.5	3.5	14	26	++
L27	1.5	2	10	28	+
L28	0.3	0.7	8	18	++
L29	2	1.2	12	15	+++

Table 2—*continued*

Protein	% of total incorporation: Intact subunit	% of total incorporation: Dissociated proteins	% of total protein lysine methylated: Intact subunit	% of total protein lysine methylated: Dissociated proteins	Exposure classification
L30	1.5	1	14	18	+++
L31†‡	0.1	0.3	—	—	+
L32‡§	1.5	1.5	—	—	++
L33	3	2	16	22	+++

* Data for S1 were extremely variable. The values given represent an average; the exposure classification is tentative.
† Fractionality of the protein is not known.
‡ Amino acid composition and molecular weight of the proteins are not known.
§ Exposure classifications for proteins L20, L31 and L32 are tentative and based on percentages of incorporation.

Table 3. *Comparison of the data on the topography of the ribosomal proteins of 30S and 50S subunits*

The trypsin digestion results are based on the data of Crichton & Wittmann (1971) and +++ in this column denotes those proteins most easily digested by trypsin. Data for S1 were not determined. The reactivity to glutaraldehyde results are from the data of Kahan & Kaltschmidt (1972). Proteins are classified in terms of the percentage concentrations of glutaraldehyde required for reaction, and the notation in this column is: +++, 0.03%; ++, 0.1%; +, 0.3%; −, did not react. Reductive alkylation results are based on the data in Table 2, but with the '+' notation scaled down to correspond with the trypsin and glutaraldehyde data: +++ in the final column denotes proteins with the highest degree of exposure.

Protein	Trypsin digestion	Glutaraldehyde reactivity	Reductive alkylation
S1	?	+++	(+++)
S2	++	++	++
S3	++	++	++
S4	+	−	+
S5	+	+	+
S6	+++	++	+++
S7	+	+	+
S8	+	+	+
S9	+	?	+
S10	++	+++	+++
S11	++	?	++
S12	+	−	+
S13	++	++	+
S14	+	+	+
S15	+	−	+
S16	+	−	+
S17	+	−	+
S18	+	++	+
S19	+++	++	++
S20	+	−	+
S21	++	++	++
L1	+++	−	+
L2	+++	−	+
L3	+	−	+
L4	+++	+	++
L5	+++	+	++
L6	+++	+	+
L7	+++	++	+++

Table 3—*continued*

Protein	Trypsin digestion	Glutaraldehyde reactivity	Reductive alkylation
L8	+++	+	+
L9	+++	+	+
L10	+++	+	++
L11	+++	+	++
L12	+++	++	+++
L13	++	−	+
L14	++	+	+
L15	++	−	+
L16	+++	−	+
L17	++	−	+
L18	+	+	++
L19	+	+++	+++
L20	++	−	+
L21	++	−	+
L22	+	+	+
L23	++	+	+
L24	−	+	+
L25	++	++	++
L27	+	+++	+
L28	+	−	+
L29	++	+	++
L30	+	++	++
L31	?	++	+
L32	+	+	+
L33	++	+++	++

Stöffler *et al.*, 1971; Zimmerman *et al.*, 1972) generally exhibit a tendency to be methylated to a lesser degree than other proteins in intact ribosomes, presumably also because of a shielding effect arising from salt linking to RNA.

The data in Table 1 indicate that all the proteins in 30S and 50S subunits react with the modifying agents, and therefore all of the proteins have at least one lysine residue at or near the surface of the ribosomes. This is the first time that evidence has been obtained that indicates that all of the proteins of ribosome subunits are at least partially accessible. If the ribosome is considered as a homogeneous unit, it would appear

from these results that all of the proteins in both 30S and 50S subunits are accessible to the surface of the subunit. However, the possibility exists that the results reported here have arisen because of the structural or conformational heterogeneity in ribosomes *in vitro*.

Consideration of the data in Table 2 leads to the conclusion that the degree of exposure of individual proteins varies considerably. However, the interpretation of the data in terms of exposed surface area of each protein must be treated with some caution, since the results are dependent on the distribution and reactivities of lysine residues in each protein. It is possible to envisage the hypothetical situation where a protein is largely exposed on the surface of the ribosome but has many of its lysine residues salt-linked to RNA, thereby yielding a protein with a low degree of alkylation which would be incorrectly interpreted as being relatively unexposed. Nevertheless, in the general case, RNA-binding proteins notwithstanding, the lysine exposure classification given in Table 2 probably gives a reasonable guide to the overall exposure of individual proteins.

The results of reductive alkylation are in very good agreement with the results obtained from the reaction of ribosomes with glutaraldehyde (Kahan & Kaltschmidt, 1972). Table 3 shows that the relative reactivities of the proteins are very similar but that the absolute reactivities differ in that some proteins in both 30S and 50S subunits did not react with glutaraldehyde, whereas all proteins were reductively alkylated.

The correlation with the trypsin-digestion data (Crichton & Wittmann, 1971) is not quite as complete as with the glutaraldehyde-reactivity data. However, the criteria involved in the glutaraldehyde reaction are very similar to those involved in reductive alkylation, whereas the action of trypsin is dependent on different criteria and involves a continuously changing environment as the digestion proceeds. Nevertheless, the correlation is reasonably good, particularly for the 30S proteins, and only for proteins L1, L2, L6, L8, L9, L16 and L19 is there a large discrepancy. Of these, the RNA-binding proteins L2, L6 and L16 would not be expected to give reliable exposure data for forming glutaraldehyde derivatives or reductive alkylation.

Immunological evidence indicates that all 30S subunit proteins are accessible to their specific antibodies (Stöffler *et al.*, 1973).

Further evidence that all the ribosomal proteins are partially exposed has been obtained by treating ribosomes with dimethyl sulphate and showing that all the proteins are modified by this reagent (G. Moore, unpublished work).

Reductive methylation offers several potential uses for future investigations on ribosome topography. The method could be applied to the determination of exposed regions of proteins by sequencing proteins derived from reductively methylated subunits and identifying which lysine residues are radioactively labelled (exposed) and which are not (unexposed). The technique may also find application in the determination of lysines involved in RNA–protein interactions by reductive methylation of isolated RNA–protein complexes, since it appears that lysine residues not involved in salt links would react preferentially with the reagent under the conditions described. In this context, it may be possible to determine which proteins of the ribosome bind mRNA, by labelling mRNA-bound ribosomes with ^{14}C-labelled reagent and free ribosomes with ^{3}H-labelled reagent and determining the $^{14}C:^{3}H$ ratio for each protein after combining the two products.

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