ANALYSIS OF KETHOXAL BOUND TO RIBOSOMAL PROTEINS FROM ESCHERICHIA COLI 70S REACTED RIBOSOMES[1]

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Received August 13,1974

SUMMARY: To investigate ribosome topography and possible function, 70S ribosomes of Escherichia coli were reacted with the dicarbonyl compound kethoxal. Ribosomal protein was extracted after reaction, and through two dimensional gel electrophoresis, the reactive proteins of the two subunits were identified. From the 30S subunit, the most reacted proteins were S2, S3, S4, S5 and S7 and from the 50S subunit, L1, L5, L16, L17, L18 and L27. The results with kethoxal are compared with other modifiers of ribosomal proteins.

INTRODUCTION

Many investigations of protein topography of Escherichia coli ribosomes have involved chemical or enzymatic modification (1-9). Only the compilation of a vast number of reagents, however, will begin to reveal some of the ribosomal surface structure, due to the specificity of different reagents and possible induced conformational changes. In this study, chemical modification was with kethoxal (β-ethoxy-α-keto-butyraldehyde), a dicarbonyl compound found to react well with proteins (10). We report here the two dimensional gel electrophoresis of ribosomal proteins extracted from 70S ribosomes reacted with kethoxal. Proteins that are reactive towards kethoxal have been identified and compared with other reagents. This work supports the conclusions from modification data with other reagents such as fluorescein isothiocyanate (3, 5) and iodination with lactoperoxidase (2) where only a few 30S and 50S proteins are highly reactive with 70S reacted ribosomes. There is also, on the whole, a surprisingly good agreement with the proteins that are readily accessible for

[1]This work was supported by U. S. Public Health Service Grant #GM20052.

modification, considering the differences in amino acid specificities of the different reagents used.

MATERIALS AND METHODS

Chemicals: ^{3}H-Kethoxal (specific activity 8 mc/mmole) was from Schwarz-Mann. Solutions for ribosome isolation were as follows: Solution I (10 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 30 mM NH_4Cl, 6 mM β-mercaptoethanol). For ribosome reactions with kethoxal and subsequent procedures, solution 1A was 10 mM Tris-acetate or -borate, pH 7-8, 10 mM MgAc, 30 mM KCl, 6 mM β-mercaptoethanol. Solution II was the same as 1A except MgAc was 0.3 mM and Solution III was the same as I except 0.35 mM NH_4Cl was used.

Ribosome Preparation and Reaction with Kethoxal: Ribosomes were prepared from E. coli strain Q13 (mid-logarithmic cells, RNase I deficient), purchased from General Biochemicals. Preparation of 70S ribosomes was according to Traub et al (11). Reactions were carried out with ribosomes in 1 ml of Solution IA. To each reaction, 0.5 ml of kethoxal (72.5 μmoles) was added and incubated at 37°C for 45 minutes. After incubation, the reaction mixture was layered over 30% sucrose in solution 1A-Borate, centrifuged, and the ribosomes resuspended and layered over sucrose an additional time to remove unbound kethoxal.

Subunit Separation, Extraction of Protein, and Electrophoresis: Kethoxal reacted 70S ribosomes were dialyzed for eight hours against Solution II with two changes. The dialyzed solution was then layered over 10-40% Solution II sucrose gradients and centrifuged at 25K for 16 hours to separate subunits. Subunits were then pelleted and resuspended in Solution 1A. Ribosomal protein was extracted by the method of Spitnik-Elson (12). Two dimensional polyacrylamide gel electrophoresis was performed as described by Kaltschmidt and Wittmam (13). Gel slabs were stained and fixed in 7% acetic acid with amido black and then destained in 7% acetic acid until clear. Protein spots were cut out and then placed in scintillation vials to dry. Gel pieces were then hydrolyzed with 0.5 ml of H_2O_2 and counted in 10 ml of Aquasol. Different gel runs varied as to the total protein added to the gel. To average the counts from different experiments, the counts for each protein were normalized to one protein (L1 for the 50S proteins and S2 for the 30S proteins) and the average value for six separate runs obtained.

RESULTS

Much work has been done on the binding of kethoxal to nucleic acids (14, 15) but only recently has it been found to react towards protein (10). From work in this laboratory, kethoxal appears to be specific for arginine and does not react with lysine or other amino acids to any significant extent (B. Steinberg, S. Dodt and N. Delihas, unpublished data).

To determine incubation times when proteins are fully reacted, the time course of reaction of ribosomes with kethoxal was followed (Figure 1). Although reaction of RNA components continues for longer times, the reaction with protein appears complete by 30 minutes. For protein topography studies, 70S ribosomes were reacted with kethoxal for 45 minutes, the subunits were separated, and

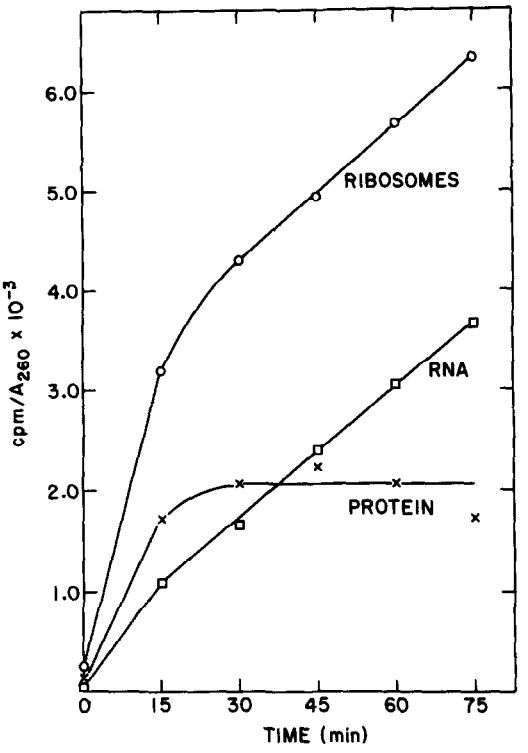

Figure 1: The time course of reaction of ribosomes with kethoxal. 70S ribosomes were incubated with kethoxal (see Methods) at 37°C for varying times and unbound kethoxal removed by repeated centrifugation through 30% sucrose. Bound kethoxal to ribosomes, RNA, and protein was related to A_{260} of ribosomes.

proteins were extracted and separated by two dimensional gel electrophoresis. 50S and 30S subunit proteins from unreacted ribosomes were also run on two dimensional gels. No differences in mobility were found in reacted proteins. Data on the average ^{3}H counts recovered in each protein spot are included in Table I. Also included in the same table is an approximate estimate of the number of arginine residues in each protein, according to the amino acid composition (16, 17) and the molecular weight (18). A comparison of the arginine content and degree of kethoxal uptake shows little or no correlation between arginine content and reactivity towards kethoxal (Table I).

The 30S subunit proteins from 70S ribosomes that are substantially reactive are in the following order: S3, S4, S5, S7, S2>S6, S13, S9, S18; the 50S subunit proteins in order of reactivity are: L1, L5, L27, L17, L16, L18>L14, L11, L19, L15, L2, L6.

TABLE I

Labeling of 30S and 50S proteins extracted from 70S ribosomes reacted with ^{3}H-Kethoxal, with corresponding number of arginine residues

Protein	^{3}H-Kethoxal bound cpm	# of Arg Residues	Protein	^{3}H-Kethoxal bound cpm	# of Arg Residues
S1	46	21	L1	930	-
S2	400	8	L2	281	19
S3	836	15	L3	193	6
S4	724	15	L4	209	12
S5	688	7	L5	766	6
S6	368	10	L6	272	7
S7	456	17	L7	100	16
S8	73	6	L8/9	236	11
S9	317	14	L10	214	9
S10	103	11	L11	314	6
S11	166	-	L12	126	16
S12	105	17	L13	142	-
S13	364	-	L14	338	-
S14	120	11	L15	304	9
S15	112	8	L16	467	13
S16	112	8	L17	687	11
S17	48	4	L18	450	10
S18	293	10	L19	306	11
S19	236	6	L20	83	13
S20	67	6	L21	108	-
S21	197	10	L22	135	-
			L23	192	6
			L24	302	6
			L25	213	-
			L26	68	4
			L27	693	7
			L28	80	-
			L29	109	5
			L30	131	6
			L31	20	-
			L32	184	-
			L33	67	7

DISCUSSION

Several conclusions can be drawn from the protein modification data shown in Table I. Most of the 50S and 30S subunit proteins react at least to some extent in 70S ribosomes and thus they must be partially exposed. Less than half of the proteins, however, react to a significant extent and only 5 30S

TABLE II

Comparison of 30S proteins reaction with kethoxal and other chemical modification reagents and enzymatic digestion

30S Protein	Kethoxal Binding	FITC[3] Uptake	Tryp[9] Digest	N-ethyl-maleimide Reaction[7]
S1	-	+++	+	+++
S2	+++	+++	+	-
S3	+++	+++	+	...
S4	+++	+++	-	++
S5	+++	-	+	...
S6	++	+	+	...
S7	+++	+++	-	...
S8	-	-	-	-
S9	++	+	-	...
S10	-	-	+	...
S11	+	-	...	+
S12	-	-	-	...
S13	++	+	-	...
S14	-	-	-	...
S15	-	+	-	...
S16	-	-	-	-
S17	-	-	-	-
S18	++	+	-	+++
S19	+	-	+	...
S20	-	+	-	...
S21	+	+++	+	++

[3]Huang and Cantor (1971)
[9]Craven and Gupta (1970)
[7]Moore (1971)

(+++) indicates strong reaction
(++) indicates moderate reaction
(+) indicates weak reaction
(-) indicates no reaction
(...) no information available

subunit proteins and 6 50S subunit proteins are very reactive. Important is the correlation with other reagents used with 70S ribosomes (Tables II and III). Included in Table III are data with modified proteins from free 50S subunits, also, since little change has been seen in comparison to reaction with 50S proteins from 70S particles (2, 5). As can be seen from Tables II

TABLE III

Comparison of 50S proteins reaction with kethoxal and other modifiers

50S Protein	Kethoxal Binding	Iodine Uptake[2]	FITC[5] Uptake	Tryp[8] Digest	Glutaral-dehyde Uptake[6]	N-ethyl-maleimide Reaction[7]
L1	+++	+	++	++	-	...
L2	++	+++	++	+++	-	+++
L3	+	+	++	-	-	++
L4	+	-	+	+++	+	...
L5	+++	+++	..	++	+	+++
L6	++	+++	..	+++	+	-
L7	-	...	+	++	++	...
L8/9	+	++	+	++	+	...
L10	+	+++	..	+++	+	++
L11	++	+++	..	+++	+	-
L12	-	...	+	++	++	...
L13	-	++	+	+	-	...
L14	++	+	..	++	+	...
L15	++	+	..	+	-	...
L16	+++	-	..	+++	-	++
L17	+++	++	..	+	-	++
L18	+++	++	..	-	+	++
L19	++	-	+	-	+++	...
L20	-	-	-	++	-	+
L21	-	-	++	++	-	...
L22	-	+	..	-	+	-
L23	+	-	+	+	+	...
L24	++	-	..	-	+	...
L25	+	+	..	++	++	...
L26	-	+++	-	+	++	-
L27	+++	+	..	-	+++	++
L28	-	++	-	-	-	...
L29	-	-	..	+	+	+
L30	-	-	..	-	++	...
L31	-	...	-	...	++	...
L32	+	++	+	-	+	...
L33	-	-	-	++	+++	...

[2]Litman and Cantor (1974)
[5]Hsiung and Cantor (1973)
[8]Crichton and Wittmann (1971)
[6]Kahan and Kaltschmidt (1972)
[7]Moore (1971)

(+++) indicates strong reaction
(++) indicates moderate reaction
(+) indicates weak reaction
(-) indicates no reaction
(...) no information available

and III, kethoxal does match some of the other reagents in the degree of reactivity of certain proteins, but there are a number of inconsistencies, also. In fairly good agreement with kethoxal from the 30S subunit are S2,

S3, S4, S6, S7, S18 and S21. The proteins from the 50S subunit showing a good correspondence appear to be L2, L5, L6, L11, L17, L18, L27 and L32.

Some useful comparisons with affinity labels and binding proteins can be made. L27 is highly reactive with modified phe t-RNA's (19, 20) and the N-bromo-acetyl analog of chloramphenicol (21). Monoiodoamphenicol (22) reacts preferentially with L16. Photoaffinity labeling of 70S ribosomes with an analog of GDP shows a strong reaction with S2 (Schrier, P., Maasen, J. and Moller, W., unpublished data). L16, L27 and S2 are proteins that are very reactive with kethoxal.

Of interest also is the correlation of the ribosomal binding proteins of antibiotics and the degree of exposure for chemical modification. S3 and S5 are reactive towards kethoxal and fluorescein isothiocyanate and also form one of the binding sites for dihydrostreptomycin (23). S5, the protein that confers sensitivity to spectinomycin and may be part of the binding site for spectinomycin (24, 25), is very reactive towards kethoxal. Interestingly enough, S12, the protein that controls the sensitivity to streptomycin, but does not appear to bind streptomycin, does not react in 70S ribosomes with kethoxal or any of the reagents shown in Table II.

Perhaps the most significant data from chemical modification studies will be with changes in modifications with and without antibiotics and other molecules that specifically interact with the ribosome. Such studies are presently being conducted.

ACKNOWLEDGEMENTS

We are grateful to Dr. Charles Cantor for the use of his laboratory for the two-dimensional gels electrophoresis and to Nancy Hsuing, David Litman and Dr. Cantor for helpful advice and discussions.

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