EFFECTS ON RIBOSOMAL ACTIVITY AND STRUCTURE OF MODIFICATION WITH SUCCINIC, MALEIC AND ACETIC ANHYDRIDES

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1. Introduction

Succinic anhydride modifies the amino groups of proteins substituting a negative charge for a positive one, which brings about a drastic change in the electrostatic properties of the molecules [1]. In proteins composed of subunits, modification is frequently accompanied by dissociation [2,3]. Therefore, treatment of ribosomes with succinic anhydride should presumably be accompanied by dissociation into different modified particles and free components. Although succinic anhydride is also able to modify tyrosine residues, above pH 5 *O*-succinyltyrosine residues are rapidly hydrolyzed with regeneration of the original tyrosine residues [4]. Maleic anhydride reacts with proteins in a way similar to succinic anhydride, but can also alkylate sulfhydryl groups as *N*-ethylmaleimide does. In contrast with succinic and maleic anhydrides, acetic anhydride reacts with amino groups without introducing negative charges.

In this paper, we report the modification of *Escherichia coli* ribosomes by succinic, maleic and acetic anhydrides. Modification is accompanied by inactivation of polyphenylalanine synthesis, peptidyl transferase and elongation factor G-dependent GTPase, and dissociation of the 70 S ribosomes into 50 S and 30 S subunits.

2. Materials and methods

Escherichia coli MRE 600 was grown, and NH_4Cl-washed ribosomes were obtained, as in [5]. Ribosomes were kept at −20°C in 5 mM Tris–HCl (pH 7.8), 20 mM magnesium acetate, 500 mM NH_4Cl, 2 mM DTT, 0.5 mM EDTA and 50% (v/v) glycerol.

Prior to treatment, 0.25 ml stored ribosomal preparation (16–20 mg ribosomes) were diluted with 100 mM Tris–HCl (pH 7.8), 20 mM magnesium acetate, 0.5 mM DTT and 0.5 mM EDTA, to final vol. 2.5 ml. To this solution the reagent was added stepwise, and the pH maintained at 7.8 by addition of 0.1 N NaOH. The treatment took place at room temperature and was completed in 1 h. When small amounts of reagent had to be added, it was dissolved in dry dioxane prior to addition. Immediately after the treatment the preparation was dialyzed at 0–5°C against 50 mM Tris–HCl (pH 7.8), 50 mM NH_4Cl, 20 mM magnesium acetate, 0.5 mM DTT and 0.5 mM EDTA, for 17 h. An untreated control was always prepared in exactly the same way as the treated preparations but without the addition of reagent. This control showed no significant changes in sedimentation or in the assayed activities with respect to the original preparation.

Ribosomal proteins were extracted with 67% (v/v) acetic acid and precipitated with 5 vol. acetone [6]. The precipitated proteins were dissolved in 10% sodium dodecyl sulphate, and the number of amino groups that had not reacted with the acid anhydride was determined with ninhydrin [7], taking molar extinctions of 67% and 100% of that of leucine for the color developed from ϵ-amino and free α-amino groups, respectively [8], and averaging the color yields of the two kinds of amino groups to 69.5%, according to the known lysine content (about 660 residues) [9] and the number of protein compo-

Abbreviations: DTT, dithiothreitol

nents (about 54) of the ribosome. Protein concentration was determined as in [10].

Poly(U)-directed polyphenylalanine synthesis was performed in a crude system containing S-100 extract [11]. Peptidyl transferase was estimated by the 'fragment reaction' assay, using C(U)–A–C–C–A-(Ac[^{3}H]Leu) and puromycin as substrates [12]. GTPase was determined by measuring the radioactivity of the inorganic phosphate liberated by hydrolysis of [γ-^{32}P]GTP in the presence of elongation factor G [13].

Sedimentation behaviour of treated ribosomes was studied by centrifugation in linear 5–20% sucrose gradients in 10 mM Tris–HCl (pH 7.8), 10 mM magnesium acetate and 100 mM NH_4Cl. The ribosomes (50 pmol) were centrifuged in a Spinco SW50.1 rotor at 48 000 rev/min for 1.25 h. The distribution of particles along the gradient was determined with an Isco density gradient fractionator.

Two-dimensional polyacrylamide gel electrophoresis of ribosomal proteins was done as in [14]. Polyacrylamide gel electrophoresis of rRNA [15] was carried out on rRNA obtained from 70 S untreated ribosomes and 50 S and 30 S particles isolated from succinylated ribosomes by sucrose gradient centrifugation.

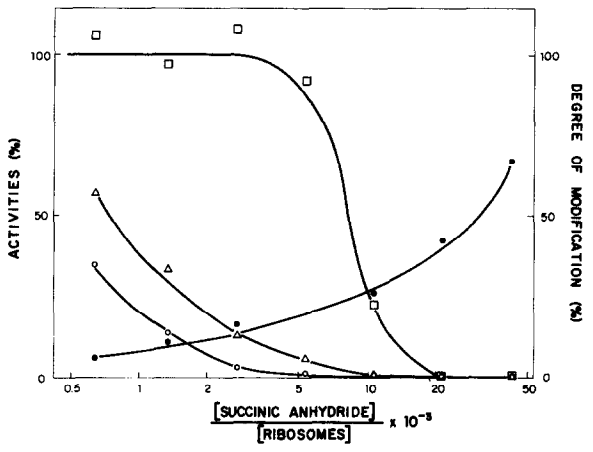

Fig.1. Modification of ribosomes by succinic anhydride and effects on polyphenylalanine synthesis (○), peptidyl transferase (□) and GTPase (△). The degree of modification (●) is expressed as the percentage of amino groups modified by succinic anhydride with respect to the total number of amino groups that react with ninhydrin in the succinic anhydride untreated control. This number agrees within 10% with the total number of protein amino groups present in the ribosome, obtained from the amino acid composition of the ribosome [9] and the number of free α-amino groups in the ribosome (714 ϵ-amino plus free α-amino groups). The ribosome activities are expressed as percentages of the corresponding activities of the untreated control. The activities of this control were: 5.6 molecules [^{3}H]phenylalanine incorporated, 3.5×10^{-3} molecules [^{3}H]leucylpuromycin synthesized and 31 molecules [γ-^{32}P]GTP hydrolyzed per ribosome.

3. Results and discussion

The degrees of modification of *Escherichia coli* ribosomes treated with different amounts of succinic anhydride and the polyphenylalanine synthesis, peptidyl transferase ('fragment reaction') and elongation factor G-dependent GTPase activities are shown in fig.1. All the activities were inactivated, peptidyl transferase being the most resistant, in agreement with results obtained with 2-methoxy-5-nitrotropone [13,16]. At a molar ratio of reagent to ribosome of about 4000, peptidyl transferase was not appreciably affected, while polyphenylalanine synthesis and GTPase were over 90% inactivated. To obtain virtually complete inactivation of peptidyl transferase the molar ratio of succinic anhydride to ribosomes had to be increased to 21 000, with modification of more than 40% of the ribosomal amino groups. Table 1 shows the effects of modification by maleic and acetic anhydrides on ribosomal activities at two different molar ratios of reagent to ribosomes. These compounds affected the three activities in a way similar to succinic anhydride, although acetic anhydride produced lower inactivation.

Modification affects mostly or exclusively the protein components of the ribosome, since the amino groups of the heterocyclic bases in mono- and oligonucleotides are not acetylated by acetic anhydride in aqueous solution, and the hydroxyl groups of ribose are only attacked under conditions stronger than those used here [17,18]. In our treated preparations no modification of r-RNA was detected using the hydroxamic reaction [18], but, because of the sensitivity of the assay, this result does not exclude modification of a low number of riboses, about 10 or less per ribosome.

Two-dimensional gel electrophoresis of the ribo-

Table 1
Modification by acetic and maleic anhydrides and effects on ribosomal activities

Reagent	Reagent / Ribosomes	Degree of modification (%)	Polyphenylalanine synthesis (%)	Peptidyl transferase (%)	GTPase (%)
Acetic anhydride	660	7.2	81	100	77
Acetic anhydride	21 000	35	< 1	41	3
Maleic anhydride	660	6.7	24	90	32
Maleic anhydride	21 000	26	< 1	18	3

The degree of modification is expressed as the percentage of protein amino groups modified by acetic or maleic anhydride. Activities are expressed as percentages of the corresponding activities of the untreated control. The activities of this control were: 2.6 molecules [^{3}H]phenylalanine incorporated, 4.7×10^{-3} molecules [^{3}H]leucyl-puromycin synthesized and 46 molecules [γ-^{32}P]GTP hydrolyzed per ribosome

somal proteins extracted from ribosomes succinylated to different extents showed a gradual disappearance of all the spots corresponding to the unmodified proteins. These results should be related to the large number of lysine residues present in the ribosome. However, proteins L2, L3, L13, L15, L17, L21, S4, S9 and S10 remained visible in the electrophoretogram of a preparation modified at a molar ratio of reagent to ribosome of 5300, and therefore seem to be more resistant than the rest.

The sedimentation patterns of preparations treated with different amounts of succinic anhydride are shown in fig.2. Modification is accompanied by disappearance of the peak of 70 S ribosomes and increases of those of 50 S and 30 S subunits (fig.2A–E). To make sure that the 50 S and 30 S peaks correspond to the subunits and not to modified ribosomes with lower rates of sedimentation produced by loosening of the structure, the RNAs obtained from the 50 S and 30 S components were subjected to acrylamide gel electrophoresis. Figure 3 shows that the 50 S and 30 S components contained almost exclusively 23 S RNA and 16 S RNA, respectively, and therefore correspond to modified 50 S and 30 S subunits. Dissociation increased with the degree of modification, being complete in the preparation treated with succinic anhydride at a molar ratio of 21 200 (fig.2E). At a higher level of modification the

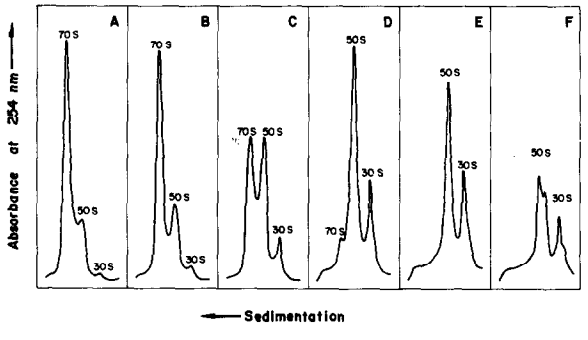

Fig.2. Sedimentation patterns of ribosomal preparations treated with different amounts of succinic anhydride. Untreated control (A) and preparations treated with succinic anhydride at the following molar ratios of reagent to ribosome: 1325 (B); 5300 (C); 10 600 (D); 21 200 (E); and 39 750 (F).

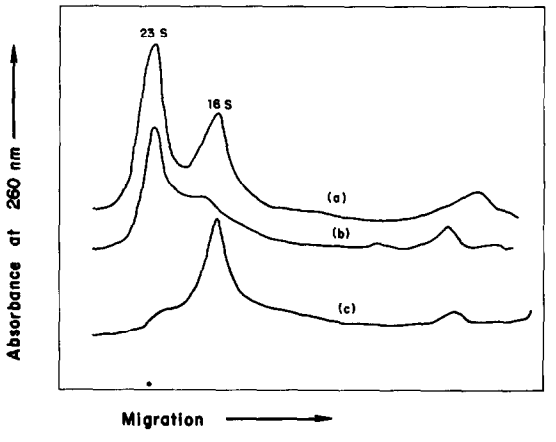

Fig.3. Densitometric tracings of polyacrylamide gels after electrophoresis of rRNA from 70 S untreated ribosomes (a) and modified 50 S (b) and 30 S (c) particles. The modified preparation was obtained by treatment with succinic anhydride at a molar ratio of reagent to ribosomes of 21 200.

50 S and the 30 S peaks are split (fig.2F), indicating the appearance of particles with lower sedimentation coefficients. These changes in sedimentation may be due to either a gross change in conformation of the 50 S and 30 S subunits, or to a loss of components by dissociation.

Ribosomes treated with acetic and maleic anhydrides were also dissociated into 50 S and 30 S subunits. For the same degree of modification acetic anhydride produced the lowest degree of dissociation.

There is no apparent correlation between dissociation and inactivation of polyphenylalanine synthesis and elongation factor G-dependent GTPase, inactivation preceding dissociation.

The present results suggest that a large part of the amino groups are not essential to the gross architecture of the ribosome; their structural role would mainly be the interaction with the solvent and the maintainance of the electric properties of the particle.

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