N-TRIMETHYLALANINE, A NOVEL BLOCKING GROUP, FOUND IN E.COLI RIBOSOMAL PROTEIN L11(o)

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SUMMARY

We show in this paper that the unknown methylated aminoacid observed in E.coli ribosomal protein L11 is N-trimethylalanine. Using [^{3}H]-alanine, we also observe the presence of N-monomethylalanine in 50S and 30S proteins whereas our previously published experiments had not detected the methylation of L33 and S11. A comparison of the relative amounts of monomethylalanine synthesized under various conditions leads us to suggest that an alternate methylation pathway, not involving S-adenosylmethionine, may exist for ribosomal proteins.

INTRODUCTION

Some E.coli ribosomal proteins are modified by post-translational methylation. This was demonstrated by in vivo or in vitro addition of radioactive methyl groups to undermethylated ribosomes synthesized by E.coli grown in the presence of ethionine (1) ; it was confirmed by growth of cultures of E.coli in a medium containing [1-^{14}C]-methionine and [^{3}H-methyl]-methionine (2,3) and by the discovery of methylation deficient mutants of E.coli (4). In every case, most of the incorporated radioactive methyl groups (75-85%) were localized in protein L11, and were found to be distributed essentially between two aminoacids : ε N-trimethyllysine,

(o) paper n°4 in the series "properties of ribosomes synthesized by E.coli grown in the presence of ethionine" ; paper n°3 is reference 1. This work will be included in a doctoral thesis(Doctorat ès Sciences) to be submitted by one of us (J-H.A.).

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and an unknown (X) which behaved as a neutral compound (1-4). We have now identified compound X as N-trimethylalanine.

MATERIALS AND METHODS

N-dimethyl aminoacids were synthesized from the [^{14}C]-labelled free aminoacids by the formaldehyde/borohydride procedure (5,6), and N-trimethyl derivatives by exhaustive methylation with methyl iodide in alkaline methanol (7). When necessary the samples were desalted on Dowex 50-X2 (8).

E.coli D10 (met$^-$) was cultivated in the presence of ethionine and the ribosomes methylated *in vivo* with [^{3}H-methyl]-methionine or *in vitro* with [^{3}H-methyl]-S-adenosylmethionine as described before (1). Proteins were hydrolysed in 5.7 N twice-distilled HCl, 0.5% mercaptoethanol at 110°C for 24 h under vacuum. Compound X was purified on a Beckman 120 C analyser, using a 55 cm column of resin AA 15, eluted with 0.2 M citrate buffer pH 3.18 at a flow rate of 70 ml/h, without ninhydrin. The radioactive fractions corresponding to compound X were desalted on Dowex 50-X2 (8) and concentrated with a rotary evaporator. Radioactivity measurements were carried out using Bray's solution in an Intertechnique SL30 scintillation counter. Descending paper chromatography was carried out using Whatman 3 MM paper at 20°C for about 18 h, with the solvent n-butanol/pyridine/acetic acid/water (15/10/3/12, v/v). Mass spectra were recorded on an AEI MS 50 instrument operating at 70 eV in the electron impact mode.

RESULTS

Compound X was found to be eluted from the aminoacid analyser 4 mn before aspartic acid, at 49 mn ± 2 mn. The absolute elution time varied from one analysis to the other, depending on the column, the solvent in which the sample was dissolved (0.1 N HCl or 0.2 M citrate buffer pH 2.2) and the amount of extraneous salt it contained. The relative positions of the peaks were, however, constant.

The following observations gave some clues which helped to restrict the choice of possible precursors. Compound X behaved as a neutral compound upon electrophoresis at pH 6.5 and 9 (1). It was thus not derived from an acidic aminoacid. Its elution position was the same whether protein hydrolysis had been carried out under oxidising conditions which completely destroyed even histidine and tyrosine (non

distilled HCl, poor vacuum) or under reducing conditions (5.7 N twice-distilled HCl + 0.5 % mercaptoethanol, 0.01 mm Hg). Its elution was similarly unaffected by acetylation in acetic anhydride/pyridine (4/1, v/v). Thus compound X had to be the derivative of a neutral non oxidizable amino-acid and could possess neither free >NH, -SH, nor -OH functions.

Reference compounds (dimethyl and trimethyl derivatives of glycine and alanine, and trimethyl valine) were accordingly synthesized starting from [^{14}C] -labelled amino acids by methods described in the experimental section. In each case a single peak was observed on the analyser after the reaction, with total disappearance of the free amino acid, and no trace of monomethyl compound (commercial samples are available, and can be located by their reaction with ninhydrin). The structures of trimethylglycine and trimethylalanine were confirmed by mass spectrometry after desalting on Dowex 50-X2, and paper chromatography (this was necessary for removing impurities that made it difficult to interpret the spectrum). Characteristic peaks were observed at m/e 72 and 58 for trimethylalanine and trimethylglycine respectively, corresponding to the ions $(CH_3)_2\overset{+}{N}= CH\text{-}CH_3$ and $(CH_3)_2\overset{+}{N}= CH_2$. Decomposition of the quaternary ammonium ions is supposed to occur even before decarboxylation and similar peaks would be expected from the dimethyl derivatives. These, however, synthesized by an unambiguous procedure (see methods), could be distinguished from the trimethyl derivatives by their chromatographic elution position. It was generally observed that each addition of a methyl group increased the acidity of the derivative on the analyser.

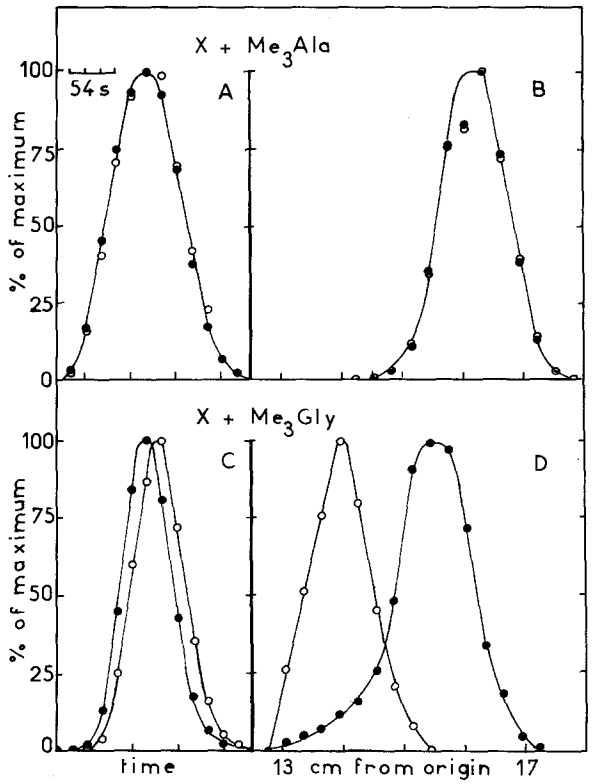

Figure 1. Chromatographic identification of compound X
A and B : comparison of [^{3}H]-X (●——●) with [^{14}C]-trimethylalanine (o——o)
C and D : comparison of [^{3}H]-X (●——●) with [^{14}C]-trimethylglycine (o——o)
A and C : elution from the aminoacid analyser. The origin of the time scale is not indicated for reasons explained in the text. Fractions of 18 sec were collected.
B and D : descending paper chromatography. Rectangles 3 mm wide were cut along the direction of migration and shaken for 30 mn in 1 ml H_2O at room temperature before counting.

Dimethylglycine, dimethylalanine, and trimethylvaline were eluted about 1.5 mn, 4 mn and 10 mn later than compound X, while trimethylglycine was eluted closer to X but at least one fraction later (i.e. 18 sec, figure 1C) and was very clearly separated from X during paper chromatography (figure 1D). Only trimethylalanine repeatedly coincided with X both on the analyser and on paper (Figure 1 A and B). Considering that the other possible di- and trimethyl aminoacids which had not been

tested were expected to elute from the analyser later than trimethylvaline, these experiments practically established the nature of compound X.

Additional evidence concerning the origin of compound X was sought by analysing ribosomal proteins from bacteria grown in the presence of [^{3}H]-alanine. Figure 2 shows that indeed a radioactive peak eluting 4 mn before aspartic acid is found in 50S but not in 30S proteins. Other peaks are also observed; their total radioactivity represents 0.3-0.4% of that present in alanine (peak not shown ; no radioactivity was found between proline and alanine) ; most of these peaks correspond to the usual aminoacids . A sizable peak, however, between serine and glutamic acid corresponds to the position of monomethylalanine; it is present in both 50S and 30S proteins. Two similar analyses carried out with ribosomal proteins prepared from cells grown in the presence of labelled proline and glycine failed to show the presence of compound X at the expected position.

In another experiment, a bacterial culture was grown in the presence of ethionine and [^{3}H]-alanine and divided into two halves. Cells from one half were immediately extracted, while those in the other half were submitted to the usual *in vivo* methylation conditions with non radioactive methionine, before extraction. Figure 3 shows that the peak which is eluted 4 mn before aspartic acid (compound X) is practically absent in 50S proteins before *in vivo* methylation and appears after methylation. On the other hand, monomethylalanine is already present before, but increases in amount after the methionine treatment. It may be noted that peak X was not detected in 30S proteins in these experiments but that, as in

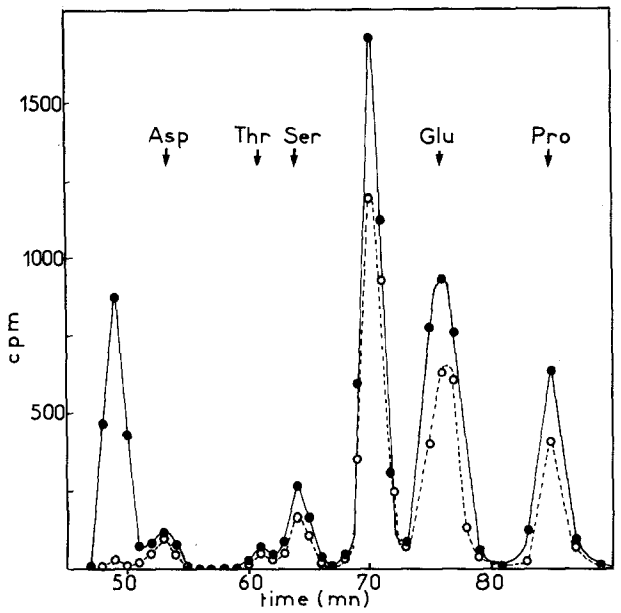

Figure 2. Amino acid elution profile of 30S and 50S ribosomal proteins prepared from E.coli D10 grown in the presence of [^{3}H]-alanine. The cells were grown at 37°C on medium 63 (16) supplemented with 0.2% glucose and 1 mM L-methionine. [2,3-^{3}H]-L-alanine (1 mCi, s.act. 40 Ci/mmole) was added at the middle of the exponential phase ; cells were harvested in late log phase. The profiles presented here correspond to the proteins from equimolar amounts of 50S and 30S particles (3.6 x 10^6 and 1.7 x 10^6 cpm in the alanine peaks respectively) although the actual analysis with 30S proteins was carried out with a larger amount of material. Fractions were collected every minute. The arrows indicate the elution position of aminoacids as judged by their ninhydrin colour in the test tubes. (●——●) 50S proteins ; (o----o) 30S proteins

the experiments of figure 2, monomethylalanine was present.

DISCUSSION

The results presented above show that compound X first observed by Alix and Hayes (1), and later by Chang and Chang (3), and Colson and Smith (4), is trimethylalanine : 1) label is found at the expected position on the analyser when E.coli is grown in the presence of [^{3}H]-alanine (figure 2) and not when the radioactive aminoacid is proline or glycine (not shown) ; 2) radioactivity introduced

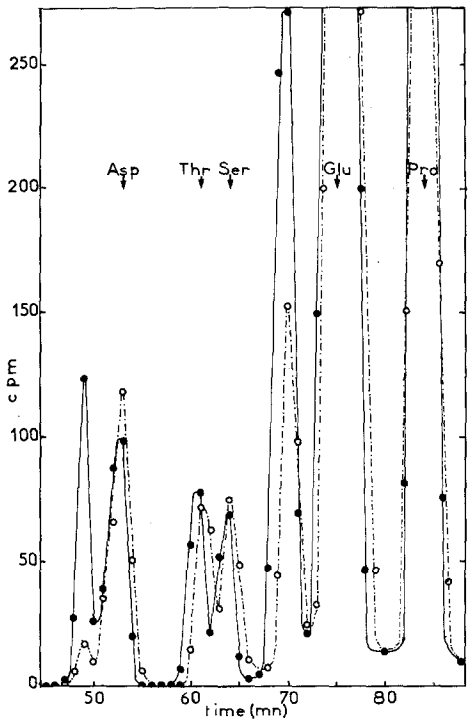

Figure 3. Elution pattern of 50S ribosomal proteins prepared from E.coli grown in the presence of ethionine and [^{3}H]-alanine before and after in vivo methylation. Cells grown in the presence of methionine until mid log phase were harvested, washed and resuspended in medium 63 (16) supplemented with 0.2% glucose. After a few minutes at 37°C, D,L-ethionine was added to a final concentration of 2 x 10^{-4} M and 15 mn later, 1 mCi [2,3-^{3}H]-L-alanine (s.act. 40 Ci/mmole) was added. Cells were harvested after two hours ; half of the culture was kept in ice, the other half was resuspended at 37°C in medium 63, containing 0.2 % glucose and 0.2 mg/ml chloramphenicol. After a few minutes, non-labelled L-methionine was added to a concentration of 10^{-4} M ; the cells were harvested after one hour. The extraction of the two batches was carried out in parallel. The total number of cpm measured in the alanine peaks (not shown) was 0.63 x 10^6 and 0.61 x 10^6 for 50S proteins after and before methylation respectively.
(●——●) 50S ribosomal proteins after methylation ; (o--o-) 50S ribosomal proteins before methylation.

into ribosomal proteins in the form of labeled alanine is found at the position of compound X only after submethylated ribosomes have been submitted to the methylation treatment (figure 3), 3) tritiated compound X and [^{14}C]-trimethylalanine migrate identically in two different systems (figure 1).

Radioactive compound X used in these experiments was always obtained from hydrolyzates of total 50S proteins but, for the following reasons, it is highly probable that trimethylalanine belongs to protein L11. First, in the present study, compound X was found to contain about 25% of the total radioactivity incorporated into 50S ribosomal proteins ; and it is known that 85% of this total is present in L11, the rest being distributed between L3 (10%) and L5 (5%) (1). Very similar distributions of methyl groups among ribosomal proteins were obtained by two other groups using different methods (3,4). Second, Chang and Chang (3) observed an elution position corresponding more or less to that of compound X for an unknown methylated compound found in L11. It is thus highly probable that trimethylalanine is the N-terminal residue of L11. The aminoacid sequence of this protein is not yet published, but it is already known that it has a blocked end group (17, and M. Dotkin, personnal communication).

In the past, a number of methylated aminoacids have been found in various proteins (12) but the modification affected side chains. Recently reports have appeared concerning α-N-methylaminoacids. Monomethylalanine has been found as the N-terminal residue of ribosomal proteins L33 and S11 and N-methylmethionine at the N-terminus of protein L16 (9-11,13). Dimethylproline was also identified as the blocking group of a cytochrome c (14). To our knowledge trimethylalanine, which was first synthesized by Fisher (15), has not previously been found in a protein.

Although the various methylation procedures did not detect the incorporation of methyl groups into 30S

proteins, monomethylalanine has been identified by other workers in S11 and L33 (9,11,13), as just mentioned. Our results concerning the presence of monomethylalanine in both 50S and 30S subunits are thus not unexpected. According, however, to the data in figure 2, there are 2 monomethylalanine residues per trimethylalanine in 50S particles, and 1.5-1.6 equivalents in 30S particles. If a full complement of completely trimethylated L11 is present in 50S subunits, it is then to be expected that another monomethylalanine-containing protein will be identified among 50S and perhaps 30S proteins.

An unexpected observation is the presence of monomethylalanine in undermethylated 50S and 30S proteins in which X is absent. *In vivo* methylation increases the amount of monomethylalanine by a factor of two in 50S proteins (figure 3) and of about five in 30S proteins (not shown). One should also note that *in vivo* methylation of ribosomes synthesized in the presence of ethionine leads to a molar ratio of mono- to trimethylalanine of 0.1 when the label comes from [^{3}H-methyl]-methionine (unpublished results) and of 3.9 when the label comes from alanine (figure 3). One should also recall that none of the methylation procedures detected incorporation of methyl groups into 30S ribosomal proteins (1-4) and that methylation of L16 was not detected by growth of *E.coli* in a medium containing [1-^{14}C]-methionine and [^{3}H-methyl]-methionine (2,3).

Perhaps all these observations have trivial explanations : presence of residual methionine during incubation with ethionine and [^{3}H]-alanine, lack of sensitivity of the

methods used for the methylation studies (1-4). It is however tempting to suggest that perhaps part of the methylaminoacids found and to be found in ribosomal proteins could arise by another route than that involving S-adenosyl-methionine.

A careful analysis of the radioactive aminoacids produced by the methylation procedure of Alix and Hayes (1) has revealed the presence, in addition to mono- and trimethyl-alanine, and εN-trimethyllysine, of three to five other radioactive components, according to whether methylation is carried out in vitro or in vivo ; the presence of some of these components has not been reported before. Their identification is being pursued and will be reported in a future paper.

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