ENDOGENOUS SYNTHESIS OF FORMYL-METHIONINE PEPTIDES IN ISOLATED MITOCHONDRIA AND CHLOROPLASTS

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SUMMARY. In the presence of a system for the incorporation of amino-acids, a formyl group donor and puromycin, mitochondria and chloroplasts can initiate "in vitro" the synthesis of peptides with formyl-methionine at the N-terminus. This indicates that in these organelles endogenous messenger(s) programme polypeptide chains starting with formyl-methionine.

Meth-$tRNA_F$, originally detected in bacteria (1, 2), is also present in mitochondria (3, 4) and in chloroplasts (5, 6). This particular tRNA has been shown to initiate polypeptide chains in prokaryotes (7, 8). It has been suggested that it is also the initiator for the prokaryotic-type organelles (3, 6, 9). The evidence for this is a) a cell-free system from Euglena chloroplasts, programmed with phage f_2 RNA, synthesized the initial peptide of the phage coat protein with formyl-methionine at the N-terminus (10), b) labeled formyl-methionine was detected in pronase digests of mitochondrial proteins from honey bees, which had been injected "in vivo" with radioactive methionine (11), and c) the *E. coli* initiation factors F_1 and F_2, required for peptide chain initiation "in vitro" with the trinucleotide AUG, are active on ribosomes from mitochondria of *Neurospora crassa* (12) and from chloroplasts of *Euglena gracilis* (13).

Experiments devised to test whether isolated mitochondria or chloroplasts can synthesize polypeptide chains with formyl-methionine at the N-terminus are described in this paper.

The results show that formyl-methionine peptidyl-puromycin derivatives can be obtained from isolated mitochondria or chloroplasts in the presence of a system for the incorporation of amino acids, a formyl

group donor and puromycin. This indicates that endogenous messenger(s) programme polypeptide chains starting with formyl-methionine. The physiological functioning of formyl-methionyl-tRNA in the initiation of protein synthesis in these cytoplasmic organelles appears therefore to be definitively demostrated.

Materials and Methods

Mitochondria were prepared (14) from Saccharomyces cerevisiae spheroplasts, obtained after digestion of the cell wall with snail gut juice. Chloroplasts were obtained from spinach leaves as previously described (6). Plate counts showed a bacterial contamination ranging from 0.5×10^3 to 5×10^3 bacteria/assay in different preparations of mitochondria and from 10^5 to 5×10^5 bacteria/assay in chloroplasts preparations (see DISCUSSION).

0.8 ml of the reaction mixture contained: chloroplasts (ca. 300 µg of chlorophyll) or mitochondria (1.5 mg of protein), 0.26 M mannitol, 50 mM HEPES pH 7.5, 20 mM mercaptoethanol, 200 µg tetrahydrofolate, 4 mM KCl, 0.04 mM EDTA, 8 mM ATP, 10 mM $MgCl_2$, 5 mM puromycin, 0.7 mM sodium formate, 45 µM methionine. When labeled, the specific activities were 210 µc/µmole for ^{3}H-formate and 615 µc/µmole for ^{35}S-methionine. Incubations were carried on at 30°C for 20 minutes and the labeling was stopped by adding cold methionine (6 µmoles) and/or formate (60 µmoles) and by chilling the tubes in an ice-water bath. The cytoplasmic organelles were disrupted by osmotic shock, by diluting the incubation mixture with low ionic strenght buffer (10 mM Tris , pH 7.5). After centrifugation at 12,000 xg, the pellet was gently resuspended and washed once with buffer. The combined supernatants were then extracted by repeated mixing on a vortex-type mixer with 1.5 volumes of ethyl acetate. The upper ethyl acetate phase, separated from the aqueous phase by centrifugation, was evaporated to dryness under N_2 stream and redissolved in 5 ml bicarbonate buffer, pH 7.5, for pronase digestion, or directly into electrophoresis of chromatography buffer. Pronase digestion was performed at 37°C for 24 hours, after addition of 0.5 umoles of unlabeled formyl-methionyl-puromycin. Labeled (^{35}S) and unlabeled formyl-methionyl-

puromycin, methionyl-puromycin and formyl-methionine were chemically synthesized (15, 16). Ethyl acetate solubility and electrophoretic mobilities of these compounds have been described by Leder and Bursztyn (16).

Results

Fig. 1 shows the results of an experiment with yeast mitochondria. Formyl-methionine derivatives are identified in the electrophoretic

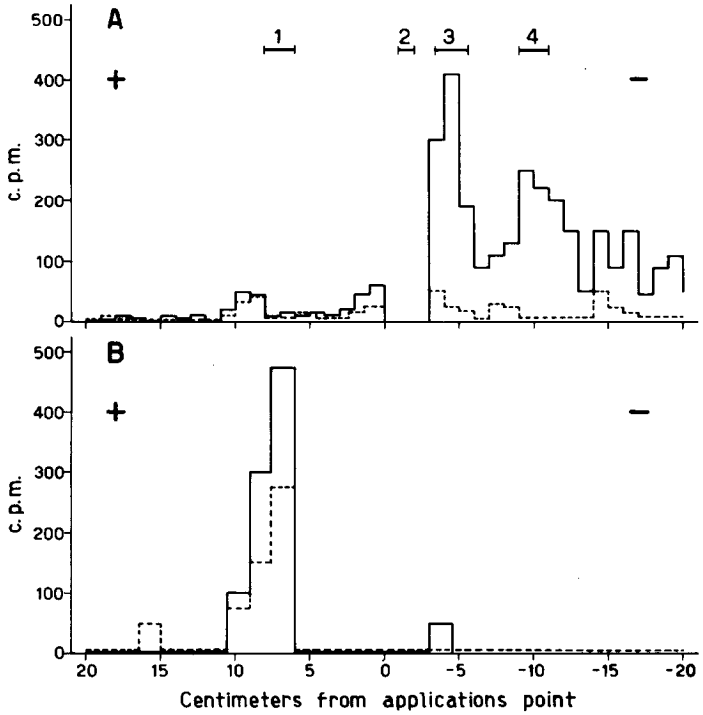

FIG. 1 Identification of the labeled formyl-methionyl-peptidyl-puromycin derivatives synthesized by isolated yeast metochondria. (see Methods for the compositions of the incubates).
Electrophoretic patterns of: A) ethyl acetate extracts from samples with (solid line = a) or without puromycin (dotted line), B) pronase digest of a; the solid line refers to an experiment in which ^{35}S-meth was the label and the dotted line to another experiment in which the label was ^{3}H-formate. In A the figures are corrected for traces of ^{35}S-meth, since the contamination was identical for both the samples. In B free ^{35}S-meth was removed by passing the digest on Dowex 50 -x 2. The arabic numerals refer to labeled reference compounds: 1) formyl-^{35}S-meth; 2) ^{35}S-meth; 3) formyl-^{35}S-methionyl-puromycin; 4) ^{35}S-methionyl-puromycin. Electrophoresis were carried out on Whatman n. 1 paper in pyridine-acetic acid-water (1/10/89), pH 3.5, at 50 volts/cm for 40 minutes at 7°C. The electrophoretograms were divided in 1 cm strips and the radioactivity measured by liquid scintillation counter.

pattern of the ethyl acetate soluble material obtained from the incubates of isolated organelles. In the presence of puromycin, the total radioactivity extracted is increased at least tenfold, and over, when corrections for traces of methionine are made. In these samples, labeled spots positively charged at pH 3.5 are evident, whereas the radioactivity in the region of formyl-methionine is negligible. These spots show the electrophoretic mobility of formyl-methionyl-puromycin and methionyl-puromycin. However, the electrophoretic analysis of a partial pronase digest of this material displayed several other labeled spots, thus indicating the presence of a family of peptidyl-puromycin derivatives.

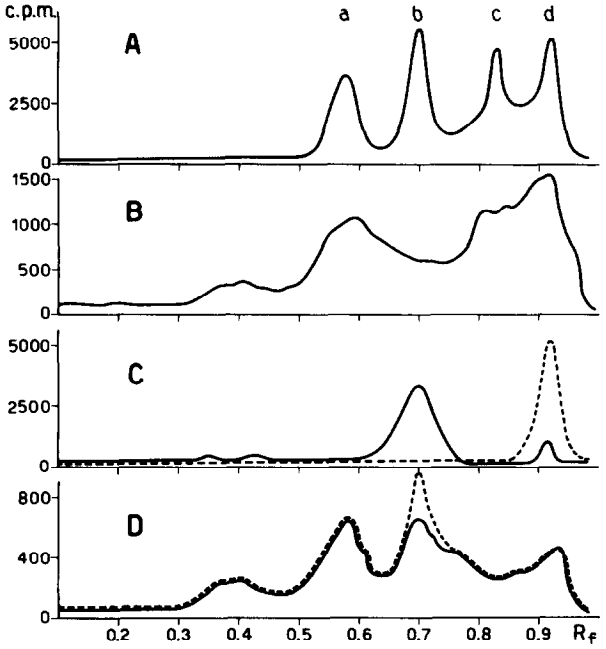

FIG. 2 Radiochromatographic analysis of ethyl acetate extractable peptidyl-puromycin derivatives synthesized by preparation of spinach chloroplasts.
A) Chemically synthesized labeled standards: a) ^{35}S-methionine, b) formyl-methionine, c) methionyl-puromycin, d) formyl-methionyl-puromycin.
B) Profile obtained from experiments with ^{35}S-methionine labeling.
C) Profile obtained from a pronase digest of chemically synthesized formyl-^{35}S-methionyl-puromycin. Dotted line: untreated sample; solid line: after the treatment.
D) Profile obtained from a pronase digest of B) (solid line); dotted line: co-chromatography with the formyl-^{35}S-methionine standard.
Ascending chromatographies were carried out on Whatman n. 1 paper, with butanol:pyridine:acetic acid:water (30:20:6:24) as a solvent.

Control experiments, in which the appearance of formyl-methionine from formyl-methionyl-puromycin was followed, showed that the commercial pronase preparation hydrolyses the aminoacid-puromycin peptide-like bond and does not contain appreciable deformylating activity. After prolonged digestion of the sample with pronase, more than 90% of the radioactivity from the peptide region was found to migrate with formyl-methionine (fig. 1 B).

The evidence for the formation of formyl-methionine derivatives has been obtained also from paper chromatography analyses. The results obtained by such a method (described in the legend) in experiments performed with isolated chloroplasts are reported in fig. 2. Again, formyl-methionine is clearly detected only after pronase digestion of the ethyl acetate soluble material.

Discussion

In experiments of incorporation with isolated mitochondria or chloroplasts, cytoplasmic and bacterial contamination must be taken into account. The possibility of some contaminant of bacterial origin as a source of formyl-methionine is excluded by the following: a) with the procedure adopted, that is ethyl acetate extraction of 12,000 x g supernatants from diluted incubates, bacteria are not recovered; b) neither peptidyl-puromycin derivatives nor formyl-methionine were detected in control experiments in which bacteria, grown by seeding plates with the incubates, were added, up to 10^9 bacteria/assay, to heat inactivated preparations of mitochondria and chloroplasts. As far as the cytoplasmic system is concerned, a significant contribution to the incorporation of formyl-methionine should be ruled out, in the light of the increasing number of papers suggesting a non-formylated or non-formylable methionyl-tRNA as initiator on 80s ribosomes (17, 18, 19, 20, 21, 22), and of the subcellular localization of transformylase, enzyme confined to cytoplasmic organelles (6, 23, 24).

More direct evidence has however been attained, as the incorporation of formyl-methionine was strongly inhibited by CAP. Further characterization is

in progress. Besides confirming the initiator role of formyl-methionyl-tRNA, these results indicate that both mitochondria and chloroplasts can initiate polypeptide synthesis "in vitro". This, if a method for the quantitative assay of initiation should become available, might represent an important finding, inasmuch as it provides the means for studying initiation in a system retaining "in vitro" a considerable number of physiological aspects.

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