PUROMYCIN DEPENDENT FORMATION OF INITIAL PEPTIDES AS A METHOD TO MEASURE THE INITIATION

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SUMMARY. Amino acid incorporating mitochondria, supplied with formyl-group donor, show a puromycin dependent formation of initial peptides. When the puromycin concentration is increased from 1 to 10 mM the length of the puromycin peptides formed is reduced up to the predominant formation of formyl-methionyl-puromycin. A method for measuring the total production of these peptides has been developed, allowing an assay of the initiation reaction. The number of initiation acts is higher than that of mitochondrial ribosomes present. Under suitable conditions the rate of formation of puromycin peptides is constant and largely accounts for the rate of synthesis of the polypeptide chains.

Previous work showed the capacity of isolated mitochondria or chloroplasts to synthesize formyl-methionyl-peptides (1). These peptides were obtained by treatment with puromycin, either as formyl-methionyl-puromycin or as formyl-methionyl-peptidyl-puromycin derivatives. Since the experimental results suggested a substantially correct functioning of the initiation mechanism even in the presence of high levels of puromycin, the possibility of the development of a method to measure the initiation reaction was suggested (1).

This paper reports experiments in which the kinetics of formation of puromycin-formyl-methionyl-peptides by mitochondria was analyzed, and a first characterization of the system was performed.

The results show that amino acid-incorporating mitochondria in the presence of high levels of puromycin can synthesize formyl-methionyl-puromycin at a constant rate. The formation of this puromycin peptide, unaffected by cycloheximide but strongly inhibited by chloramphenicol, requires ATP and N^{10}-formyl-tetrahydrofolic acid (N^{10}-formyl-THFA). Under experimental conditions corresponding to the maximum rate of synthesis, the incorporation of other amino acids in initial peptides, if any, is strongly reduced and the formation of methionyl-lacking peptides is absent.

MATERIALS AND METHODS

Preparation of mitochondria from lactate-grown Saccharomyces cerevisiae and the degree of bacterial contamination of the incubates have been previously reported (1). In the present experiments 1 ml of the reaction mixture contained: mitochondria (500 µg of proteins), 0.3 M mannitol, 70 mM Hepes pH 7.5, 5 mM mercaptoethanol, 15 mM KCl, 5 mM orthophosphate, 20 µM EDTA, 5 mM ATP, 8 mM Mg acetate, 2 mM GTP, 20 mM PEP, excess of pyruvate kinase and a mixture of cold amino acids 20 µM each, with the exception of the labelled amino acid(s). The source of cold formyl-group was N^{10}-formyl-THFA (80 µM) obtained through acid-base treatment of commercial leucovorin (N^{5}-formyl-THFA) (2). The sources of labelled formyl-groups were or N^{10}-^{14}C-formyl-THFA (80 µM, 57 µCi/µmole), previously enzymatically synthesized and purified on DEAE column (3), or ^{14}C-formate (1 mM, 57 µCi/µmole) and THFA (80 µM) directly added to the incubate. At the end of the incubation the samples, adjusted to pH 8.2, were extracted three times with 3 volumes of ethyl acetate (4) and the radioactivity incorporated in puromycin peptides was evaluated by scintillation counting of combined ethyl acetate phases. Using this procedure the recovery of formyl-methionyl-puromycin is more than 95%, and further extractions did not increase appreciably the recovery of other labelled peptides. The significant amount of contaminating radioactivity, present in the ethyl acetate extracts even in experimental 0 time and in the samples without puromycin, can be drastically reduced by a repeated ethyl acetate washing of the labelled amino acid(s) before adding them to the incubate.

RESULTS AND DISCUSSION

The experiments mentioned above suggested that puromycin at levels inducing a rapid and complete block of the chain elongation does not inhibit the initiation mechanism. A mitochondrial protein-synthesizing system, in which labelled methionine is added after the puromycin-induced release of the growing peptides, should measure the methionine incorporation only in the N-terminal position of puromycin peptides formed through a new initiation act. Since the rate of formation of these initial peptides can be measured as ethyl acetate-extractable puromycin derivatives, this system allows an assay of the initiation reaction. Thus the experimental plan followed took in account a short pretreatment of the mitochondria with puromycin before the addition of labelled methionine.

Table 1 shows experiments in which the effects of different puromycin concentra-

TABLE 1. ACTION CURVE OF PUROMYCIN ON FORMATION OF THE FORMYL-METHIONYL-PEPTIDYL-PUROMYCIN DERIVATIVES IN MITOCHONDRIA

	n moles incorporated (Δ on 0 time)/20 min/mg protein		
Puromycin	^{14}C-formyl-group	^{35}S or 1-^{14}C methionine	^{14}C other amino acids
no addition	3.0	5.0	-
1 mM	5.8	-	-
2.5 mM	13.3	10.0	5.1 (^)
4 mM	-	14.8	3.2 (^)
8 mM	35.0	42.0	not measurable (+)
8 mM	42.7	49.4	not measurable (++)
10 mM	52.7	50.0	not measurable (+++)

For incubate composition and for the source of formyl-group see Materials and Methods. ^{35}S (615 μCi/μmole) or 1-^{14}C (57 μCi/μmole) methionine 30 μM.
(^) Average value for a mixture of the following amino acids: Ala, Glu, Ile, Pro, Tyr, Arg, Gly, Lys, Val, Ser, Asp, Phe, Thr, Leu: each 10 μCi/μmole, 20 μM.
(+) 1-^{14}C leucine 62 μCi/μmole, 30 μM. (++) U-^{14}C valine 230 μCi/μmole, 30 μM. (+++) U-^{14}C serine 151 μCi/μmole, 20 μM.
The labelled compound(s) was added after 7 min of puromycin pretreatment of the mitochondria incubated in complete medium.

tions on the incorporation in puromycin peptides of formyl-group, of methionine and of other amino acids were investigated. The aspects most worthy of note appear to be the following: a) the molar ratio between the ^{14}C-formyl-group and methionine incorporated results close to one for every concentration of puromycin tested; b) by increasing the concentration of puromycin from 1 to 10 mM the incorporation of both formyl-group and methionine is stimulated, while, on the contrary, the incorporation of other amino acids is strongly reduced; c) the concentration of puromycin most effective in the formation of labelled peptides is much higher than that required to obtain a complete inhibition of protein synthesis and a rapid release of polypeptides, both "in vitro" and "in vivo" systems (5, 6, 7, 8, 9, 10).

The ratio between formyl-group and methionine, incorporated in these puromycin derivatives, indicates that methionine occupies only the N-terminal position and that these peptides are initial.

The decrease in incorporation of amino acids other than methionine by increasing puromycin concentration might be explained by the formation of shorter

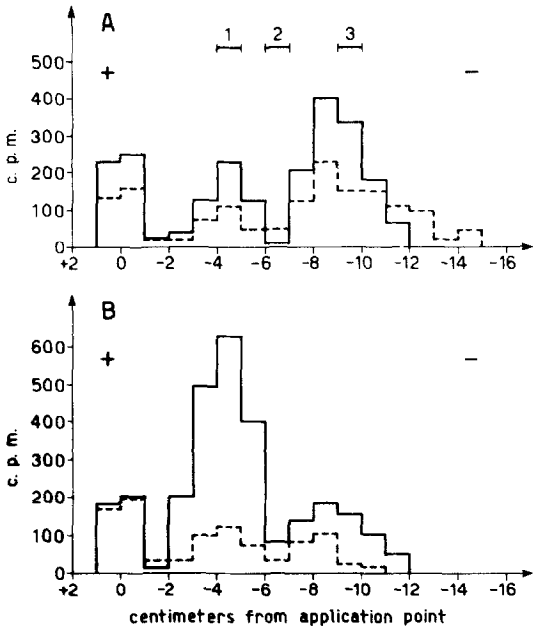

Fig. 1. Electrophoretic patterns of the labelled puromycin derivatives formed by isolated mitochondria. The graphs are corrected for contaminating radioactivity present in the 0 time of incubation.
Solid line: the label was 1-^{14}C methionine 57 μCi/μmole, 30 μM.
Dotted line: the label was a mixture of ^{14}C amino acids (see table 1).
A) 3.5 mM puromycin; B) 8 mM puromycin.
Electrophoresis were carried out on Whatman n. 1 paper in formic acid-acetic acid buffer pH 1.8 at 50 volts/cm for 80 min at 7°C. The arabic numerals refer to chemically synthesized labelled reference compounds: 1) formyl-methionyl-puromycin; 2) methionine; 3) methionyl-puromycin.

chains, in spite of a lack of a direct evidence that methionine peptides contain also other amino acids. This hypothesis is suggested also by the electrophoretic patterns of peptidyl-puromycin derivatives formed at different concentrations of puromycin in the medium (Fig. 1). At 3.5 mM puromycin two methionine labelled spots appear: the former shows the electrophoretic mobility of the formyl-methionyl-puromycin, the latter is probably due to a partial overlap of different compounds, and it is evident also in electrophoretic analysis of the products obtained by identical experiments in which other labelled amino acids substituted for ^{14}C-methionine. As previously reported (1) a partial pronase digest of this methionine-labelled material displayed several other spots, and a complete digestion released the bulk of radioactivity as formyl-methionine. Thus, formyl-methionyl-puromycin is not the only initial puromycin peptide synthesized, but formyl-methionyl-aminoacyl-puromycin derivatives can be obtained. At 8 mM

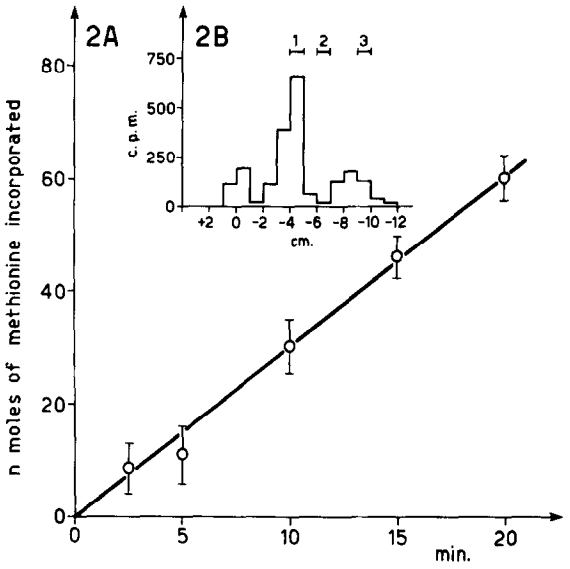

Fig. 2A. Paper electrophoresis of ethyl acetate extract from isolated mitochondria incubated with 10 mM puromycin for 20 minutes.
Fig. 2B. Time course of initial puromycin peptides formation by isolated mitochondria. Complete medium with 10 mM puromycin.

puromycin the production of formyl-methionyl-puromycin rises, while the synthesis of other formyl-methionyl-peptides seems to be repressed. Thus, formyl-methionyl-peptides of different average length are synthesized at different concentration of puromycin. A spot at the origin is also present in both the electropherograms. However the bulk of this material does not correspond to another class of compounds: in fact, when eluted and reelectrophorated, this spot originates the same spots as described above.

The kinetic of formation of formyl-methionyl-peptides was analyzed in the presence of 10 mM puromycin. At this concentration of the inhibitor, corresponding to almost exclusive production of formyl-methionyl-puromycin (Fig. 2A), the rate of incorporation of methionine was constant up to at least 15 minutes (Fig. 2B).

This finding allowed the comparison between the rate of incorporation of methionine as NH_2-terminal end and the rate of incorporation of methionine in internal position of the polypeptide chains synthesized by mitochondria (Table 2). Although the amino acid composition of the synthesized protein is unknown, the data are suitable for interesting considerations. Assuming a value of 3% for the frequency of methionine residues in the polypeptides (11) and taking the incorporation of methionine in the samples without puromycin as a measure of the rate of elongation (as it seems likely that the initial methionine is largely cleaved off), we obtain a potential

TABLE 2. RATE OF METHIONINE INCORPORATION IN PROTEINS AND OF PEPTIDYL-PUROMYCIN DERIVATIVES FORMATION IN ISOLATED MITOCHONDRIA

	n moles of methionine incorporated/20 min/mg mitochondrial RNA			
	protein fraction (x)	inhibition %	puromycin peptides (xx)	inhibition %
Control	380	-	930	-
CAP 30 uM	170	55	320	66
CHI 12 uM	370	-	960	-

(x)Mitochondria were incubated in complete medium without puromycin. The incorporation was stopped by addition of one volume of 0.1 M cold methionine. After 5 min at 0°C the mitochondria were centrifuged down, the pellet hydrolyzed 20 min at 95°C in 5% CCl_3COOH and insoluble material collected on filter. (xx) 10 mM puromycin was present in the incubates. The samples were processed with ethyl acetate technique (see Materials and Methods). CAP: chloramphenicol; CHI: cycloheximide.

capacity of one initiation reaction against about thirty amino acid residues polymerized. When the number of initiations (n moles of methionine incorporated in puromycin peptides) is correlated with the number of mitochondrial ribosomes (estimated by RNA content) our data show that the initiation process has been repeated two or three times for each ribosome (considering all the ribosomes as fully active).

The action mechanism of puromycin in these experiments is not clear. Polyribosome disaggregation and concomitant partial release of pieces of the protein synthesis machinery as a result of the supply of this inhibitor have been already described (5, 6, 7, 8, 12). It is reasonable to suppose that an additional effect of high level of puromycin consists in a similar but more rapid process. As far as this point is concerned a more detailed discussion, together with a study of ribosomal system behaviour, will be the object of a next paper.

The production of formyl-methionyl-puromycin is known to occur to an appreciable extent in several kind of bacteria (13), in chloroplasts of Acetabularia (14) and of Euglena (personal observation), in mitochondria of HeLa cells (15), besides in isolated mitochondria and chloroplasts. Moreover, methionyl-puromycin and methionyl-valyl-puromycin are detectable as products synthesized by intact reticulocytes (to be published).

These final considerations, together with the above-reported formation of formyl-methionyl-peptides at a constant rate, seem to strengthen the possibility of employing the puromycin dependent formation of initial peptides as a method to measure the total potential activity of the initiation mechanism in natural systems.

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