NH_2-TERMINAL METHIONINE IN NASCENT PEPTIDES FROM *NEUROSPORA CRASSA*

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SUMMARY

NH_2-terminal amino acid analysis of mature proteins from mycelia of *Neurospora crassa* reveals the absence of methionine and presence of glycine, alanine, and serine as major amino-termini. Examination of short nascent peptides by a variety of methods shows the presence of methionine at the NH_2-terminal position. This observation suggests that methionine is cleaved during the maturation process. It is concluded that methionine is an initiating amino acid in this eukaryote, although other modes of initiation have not been eliminated.

Since Marcker and Sanger (1) discovered that formylmethionyl-tRNA (fMet-tRNA) initiates protein synthesis in bacteria (2,3) an extensive search for the initiating tRNA has been made in various eukaryotes. The prokaryotic-type organelles (21,25) and bacteriophages (R_{17}, f_2) (23, 24) have mechanisms of initiation similar to those of *E. coli*. The N-acetylated amino acids such as N-acetyl-valine (16), N-acetyl-serine (17), and N-acetyl-glycine (18) have been suggested as initiating amino acids in eukaryotes. Caskey (26), and Marcker's group (5,19) have reported two species of methionyl-tRNAs from eukaryotes, one of which (Met-$tRNA_{F*}$) is readily formylated by *E. coli* enzymes. The latter group concluded that $tRNA_{F*}^{Met}$ is an initiator tRNA on 80S ribosomes. Jackson and Hunter (6), and Wilson (8) have supported this view with their studies involving hemoglobin synthesis.

In this study with the fungus *Neurospora crassa*, we observed that methionine appeared in nascent peptides, but not in the mature proteins in the NH_2-terminal position.

MATERIALS AND METHODS

Preparation of cell free extracts — The wild type strain of *Neurospora crassa* (74-OR23-1A) was grown in Vogel's liquid sucrose medium (27) at 30°C

for 12 hours with aeration. Mycelia were harvested and washed with sterile water and 0.01 M Tris-HCl buffer, pH 7.5, containing 0.005 M $MgCl_2$, 0.06 M KCl,and 0.06 M mercaptoethanol. Washed mycelia were ground by a Bronwill homogenizer and buffer was added equivalent to two times the wet weight of mycelia. The S-30 fraction was obtained by centrifuging the crude extract twice at 30,000 x g for 20 min. The upper two-thirds of the supernatant fraction was saved, dialyzed against the buffer, and used for NH_2-terminal analysis, as well as amino acid incorporation experiments.

Analysis of NH_2-terminal amino acids by the dinitrophenyl method — DNP-end groups were identified and estimated by the procedure of Frankel-Conrat, Harris and Levy (13) and Waller and Harris (14). A portion of crude extract was centrifuged for three hours at 105,000 x g for separation of a ribosomal pellet from soluble proteins. The NH_2-terminal analysis was carried out with all three fractions, crude, ribosomal, soluble as well as fractionated soluble protein. Detailed procedures will be published elsewhere.

Isolation of ribosomes in a post-initiation state — The reaction mixture for endogeneous mRNA-directed protein synthesis contained the following reagents; Tris-HCl 25 mM, pH 7.5; KCl, 60 mM; $MgCl_2$, 5 mM; ATP, 1 mM; GTP 0.1 mM; phosphoenolpyruvate, 5 mM; pyruvate kinase, 20μg/ml; a single ^{14}C-amino acid (5 μc/ml of Schwarz's StanSTAR, 50 mc/mMole) for each individual experiment plus 19 cold amino acids, 005 mM; S-30 fraction, 0.5 ml/ml reaction mixture. The reaction mixture was incubated for 5-7 min at 33°C and the reaction stopped by adding cold buffer containing actidione, 0.1 mg/ml. Ribosomes in a post-initiation state were pelleted from the reaction mixture by centrifugation through 1 M sucrose containing buffer and actidione. To obtain ribosomes carrying mainly short nascent peptides from intact cells, twelve hour old mycelia grown in Vogel's minimal media containing 10^{-4} x 5 M cold amino acids were transferred to fresh minimal media. After incubation for 15 min to partially deplete the free-amino acid pool, the required ^{14}C-amino acid (15 μc/10 g mycelia/100 ml) was added and incubated 5 min. Further uptake of ^{14}C-amino acid was stopped by the addition of cold water containing actidione.

Amino-termini of short nascent peptide and peptidyl-puromycin — Edman degradation of short peptide material was carried out by the method of Gray (11). (see Tables). Peptidyl-puromycin was obtained by the method of Leder and Bursztyn (12).

TABLE I

The average NH_2-terminal residues of the proteins from various fractions of Neurospora mycelia and a crude extract of E. coli B.

NH_2-terminal Amino Acids	Average values from various mycelial fractions		E. coli B crude	
	mμ moles	%	mμ moles	%
Glycine	1,145	35.0	20.0	3.2
Alanine	770	21.0	130.6	21.2
Serine	493	12.0	165.9	26.9
Threonine	306	9.0	36.9	6.0
Valine	303	8.8	-----	----
Aspartic Acid	204	7.0	17.9	2.8
Glutamic Acid	181	6.0	30.0	4.8
Lysine	56	1.0	-----	----
Phenylalanine	----	-----	-----	----
Methionine	----	-----	261.3	42.3

RESULTS

NH_2-terminal amino acid of mature proteins — Table I shows the average percent of each DNP-amino acid from crude, ribosomal, and soluble fractions. Glycine was found most frequently in the NH_2-terminal position of all mature mycelial proteins. The three major NH_2-termini were glycine, alanine and serine. As a control, the total crude extract of E. coli B was analyzed for the NH_2-terminal residues by the present method. These results in Table I are similar to those obtained by Waller (14) for E. coli B. There is a significant difference in the percentages of specific amino acids in the NH_2-terminal position for these two different organisms. We selected five different

TABLE II

Edman degradation of C^{14} Amino Acid labeled short nascent peptides, and peptidyl-puromycin from a cell-free system.

Amino Acids	short nascent peptides from ribosome [a]	peptidyl-puromycin [b]
	Counts/min/assay	Counts/min/assay
Glycine	3,988	2,460
Alanine	3,128	10,240
Serine	9,543	2,600
Phenylalanine	4,917	----
Methionine	20,769	41,820

[a] Edman degradation of short peptides was carried out by the following procedures; the isolated ribosome was treated with phenol to extract RNA, and treatment with 1 per cent trimethylamine to strip the peptides from tRNA. After one cycle of Edman degradation method of Gray (11) the released phenylthiohydantoin was extracted into butyl acetate. The butyl acetate extracts and aqueous fractions were dried, and transferred to counting fluid (20). The efficiency ranged from 50-80%. [b] The preparation of peptidyl-puromycin followed the method of Leder (12). After incubating the reaction mixture for 5 min with ^{14}C-amino acid, puromycin was added to the mixture to a final concentration 10^{-4} M and incubated for an additional 10 min. The pH was adjusted to 10 with 0.5 M sodium carbonate-bicarbonate, pH 10. The buffered mixture was then extracted twice with equal volumes of ethyl acetate. The combined extract was washed with a small amount of water and dried for Edman degradation. The yield of the degradation was 70-90%.

amino acids (Gly, Ala, Ser, Phe, and Met) for NH_2-terminal amino acid analysis of short nascent peptides. Glycine, alanine, and serine appeared as the three major amino acids (up to 70%), and phenylalanine and methionine were not detected at all in the mycelial fractions.

Amino-terminal analysis of the short peptides — Table II shows the results of the NH_2-terminal analysis of short nascent peptides obtained from ribosomes and as peptidyl-puromycin products in vitro. In both experiments, methionine was found most frequently in the NH_2-terminal position of growing

peptides in mycelia. NH_2-terminal analysis of the short nascent protein from intact cells again shows methionine as a frequent NH_2-terminal amino acid (Table III). Another experiment performed with ^{35}S methionine provided a check of the radioactivity contributed from ribosomal or soluble RNA (Table III). The various methods showed methionine to appear with high frequency as the NH_2-terminal amino acid of nascent proteins while glycine appeared most frequently in this position in mature proteins.

TABLE III

Edman degradation of ^{14}C-Amino Acid labeled nascent peptides from intact cells.

Amino Acid	Short nascent peptides from ribosomes	
	Experiment a	Experiment b
	Counts/min/assay	Counts/min/assay
Glycine	880	165-^{14}C
Alanine	660	---
Serine	1,620	---
Phenylalanine	500	---
Methionine	2,400	1,230-^{35}S

a) Labeled short nascent peptides from intact cells were prepared by the following procedures: Ribosomes by homogenization were pelleted through 1M sucrose, treated with phenol and 1% triethylamine. The yield of Edman degradation was rather low, particularly with methionine (40 - 50%).

b) Another experiment with methionine-^{35}S was carried out with intact cells. the efficiency of degradation was 79 - 89%.

DISCUSSION

In bacteria the initiation of protein synthesis depends on the incorporation of formyl-methionine, a methionine residue with its amino group blocked (1-3, 21). The formyl-methionine is carried to the ribosome by a unique species

of tRNA which can recognize the initiator codon sequence and allow the formylation of the methionine residue it carries. After or during the protein synthesis the formyl group is removed enzymatically, and sometimes the methionine residue is trimmed off as well (22). The initiation of protein synthesis in bacteriophages, R_{17} and f_2 (23, 24), and prokaryotic-type organelles (21,25) follows the bacterial mode.

Caskey et al. (26) have found two methionyl-tRNAs from guinea pig liver, only one species of which is readily formylated by E. coli transformylase. They also confirmed the absence of a transformylase in the cytoplasm. Since this report, there have been many suggestions of possible initiating amino acids from eukaryotes including N-acetyl-valine (16), N-acetyl-serine (17), and N-acetyl-glycine (18). Marcker's group (5, 19) have alternatively suggested that eukaryotic cells contain two species of methionine accepting tRNA. They propose that one ($Met\text{-}tRNA_{F^*}$) incorporates methionine only into the N-terminal position of polypeptides whereas the other ($Met\text{-}tRNA_{M^*}$) dontates methionine into internal positions further suggesting that $tRNA_{F^*}^{Met}$ is an initiator tRNA on 80S ribosomes, Jackson and Hunter have investigated the role of methionine in the initiation of hemoglobin synthesis (6). They showed that the α and β chains of rabbit globin are initiated by the incorporation of a methionine residue in the N-terminal position. This methionine is removed at an early stage of chain growth.

Whether such a trimming mechanism is operative in other eukaryotes remains an open question. Analysis of the NH_2-terminal residues of a complex mixture of mature proteins from Neurospora crassa reveal that the end groups do not reflect a random distribution of amino acids. Glycine, alanine, and serine account for about 70 per cent of the observed amino-terminal residues. These observations are significantly different from E. coli (14). Particularly, methionine is absent from NH_2-terminal positions in mycelia proteins of Neurospora crassa. We have examined the NH_2-terminal of short nascent protein by various methods both in vitro and in vivo. The results by Edman degradation showed that methionine appeared frequently at the NH_2-terminal position. This observation suggests that methionine is cleaved during the growth of peptides. It is further suggested that protein synthesis of Neurospora crassa is initiated with methionine. Epler (25) has investigated formylatability of tRNAs of mitochondria and cytoplasm, the latter being only weakly formylated by E. coli enzymes. We do not rule out the possiblity that certain proteins may

be initiated by derivatives such as N-acetyl amino acid. Experiments are in progress to determine whether there is a unique methionyl-tRNA in this eukaryote.

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