Initiation of Protein Synthesis II
A Convenient Assay for the Ribosome-dependent Synthesis of N-formyl-C^{14}-methionylpuromycin*

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One of the early steps in the initiation of protein synthesis in bacteria, following binding of met-tRNA** to the initiator codon-ribosome complex and its formylation is the transfer of N-formyl-methionine into the initial peptide bond. Formylation of the formyl-accepting species of met-tRNA (Clark and Marcker, 1966; Kellogg, Doctor, Loebel and Nirenberg, 1966) appears to facilitate this initial transfer reaction according to recent studies of a chemically analogous reaction in which the ribosome-dependent synthesis of N-formyl-met-puromycin was observed (Bretscher and Marcker, 1966; Zamir, Leder and Elson, 1966). The puromycin reaction, in general, has been studied as an analogue of peptide bond formation in protein synthesis (Nathans, 1961, Smith et al., 1965), but formation of N-formyl-met-puromycin is particularly pertinent to the study of the initiation of protein synthesis as well as to the elucidation of the mechanism of peptide bond formation. Since a convenient method for detecting the formation of a single and, in this case, the initial peptide bond has not been available, we have devised and validated a simple assay, described below, which measures formation of such a bond by specifically detecting N-formyl-C^{14}-met-puromycin.

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**The following abbreviations have been used: Met, methionyl, met-tRNA, methionyl-transfer RNA; A, U and G refer to adenine, uridine and guanosine respectively. Internal phosphodiesters, indicated by p as in ApUpG, are (3'-5'). The left hand side of oligonucleotides as written corresponds to the 5' end.

METHODS

Materials - Preparation and characterization of E. coli B N-formyl-C^{14}-met-tRNA, 0.5 M NH_4Cl washed E. coli MRE-600 ribosomes and the trinucleoside diphosphate, ApUpG, have been described elsewhere (Leder and Bursztyn, 1966a). N-formyl-methionine was synthesized according to Sheehan and Yang (1958). N-formyl-met-puromycin was synthesized by shaking a 0.3 ml anhydrous N,N-dimethyl formamide reaction mixture containing 100 µmoles of puromycin (Nutritional Biochemicals Corp.), N-formyl-methionine and dicyclohexylcarbodiimide for four hours at 4°. The reaction mixture was evaporated to dryness under reduced pressure. The reside was suspended in 0.5 ml 0.1 M sodium acetate, pH 5.5, and N-formyl-met-puromycin was extracted with two 1.0 ml portion of ethyl acetate. Met-puromycin was formed by removal of the formyl group from N-formyl-met-puromycin by incubation in acidic methanol (Sheehan and Yang, 1958).

Analysis - Electrophoresis was carried out on Whatman 3 MM paper in 0.05 M pyridine acetate buffer, pH 3.5, at 30 volts/cm for 45 minutes at 4°. Compounds containing free α-amino groups were detected with 0.25% ninhydrin-acetone spray; those blocked in the α-amino group, by the method of Zahn and Rexroth (1955). Radioactive materials on electrophoretograms were detected by cutting one cm lengths from paper strips and determining the radioactivity in the liquid scintillation counter.

Assay - The incubation was carried out in two stages. This first stage comprised the conventional ribosomal binding system (Nirenberg and Leder, 1964) in which the binding of N-formyl-C^{14}-met-tRNA, in the presence of ApUpG, was allowed to proceed to completion. Each 0.05 ml first stage reaction mixture contained: 0.1 M tris-acetate, pH 7.2; 0.05 M KCl, 0.01 M magnesium acetate; 0.15 A^{260} units ApUpG; 7.6 µµmoles N-formyl-C^{14}-met-tRNA (specific activity, 198 µc/µm); and 3.0 A^{260} units of ribosomes. Incubation was at 37° for five minutes whereupon tubes were placed in an ice-water bath and chilled for two minutes. The second stage of incubation was initiated by the addition of 0.05 µmoles puromycin diHCl contained in 0.005 ml of water to the indicated reaction mixture. Incubation was then carried out at 37° for 30 minutes at which time the reaction is essentially complete. N-formyl-C^{14}-met-puromycin formed was then detected by the addition of 1.0 ml of 0.1 M sodium acetate, pH 5.5, to each reaction mixture followed by 1.5 ml ethyl acetate. The reaction mixtures were extracted by ten, five second long agitations on a vortex-type mixer. The upper ethyl acetate phase was completely separated from the lower aqueous phase by brief (less than 30 seconds) centrifugation in a desk-top International Clinical Centrifuge. One ml of the ethyl acetate phase was then added to 10 ml of Bray's solution for liquid scintillation counting (Bray, 1960).

Ethyl acetate solubility - The amounts of various C^{14}- or H^3-methionine derivates extracted in ethyl acetate from aqueous solutions were determined as a function of pH by the addition of 10 µµmoles of each compound to 1.0 ml of each of the following buffers: 0.1 M sodium acetate, pH 4.0 and 5.0; 0.1 M sodium phosphate, pH 6.0, 7.0 and 8.0; and 0.1 M sodium carbonate - bicarbonate, pH 9.0 and 10.0. The buffers were then extracted with 1.5 ml of ethyl acetate and the amount of radioactive compound contained in a 1.0 ml aliquot was determined as described above.

RESULTS

Reaction mixtures containing partially formylated C^{14}-met-tRNA give rise to free C^{14}-methionine and certain of its small molecular derivates in the course of the transfer of N-formyl-C^{14}-methionine from tRNA into the initial peptide bond. Among these, N-formyl-C^{14}-methionine and C^{14}-methionine are produced by non-specific hydrolysis of the corresponding aminoacyl-tRNA. The ethyl acetate solubility of N-formyl-C^{14}-met-puromycin and of several derivatives of radioactively labeled methionine are shown in fig. 1. Approximately 50% of the 10 µµmoles of carboxyl - and amino-blocked N-formyl-C^{14}-met-puromycin added is extracted in the one ml aliquot of the ethyl acetate extract throughout the range of pH's tested (4.0-8.0). C^{14}-methionine is not extracted in ethyl acetate,

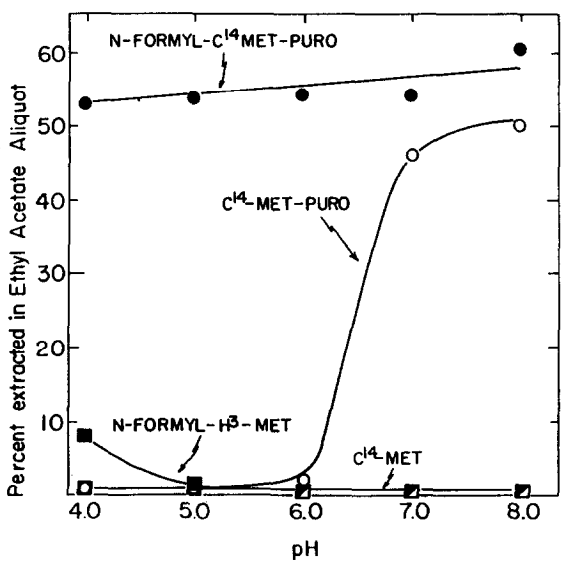

Fig. 1. Ethyl acetate extraction of labeled methionine derivatives as function of pH. Conditions of extractions are described in the text. Symbols represent the following; ●——●, N-formyl-C^{14}-met-puromycin; 0——0, C^{14}-met-puromycin, ■——■, N-formyl-H^3-met; □——□, C^{14}-met.

having both a dissociable carboxyl and proton accepting amino group. N-formyl-H^3-methionine is not extracted at pH's above 5.0, having a dissociable carboxyl group. C^{14}-met-puromycin, having a proton accepting amino group, is extracted only at pH's above 6.0. Therefore, at pH 5.5, N-formyl-C^{14}-met-puromycin may be selectively extracted from aqueous solutions contaminated by any or all of the above compounds. The assay may be altered to select for C^{14}-met-puromycin or certain other unblocked peptidyl-puromycin derivatives at pH 8.0, though blocked derivatives would also be extracted.

Results of typical assay, based on the selective ethyl acetate extraction of N-formyl-C^{14}-met-puromycin, are shown in table 1. In the absence of puromycin, background extraction of ethyl acetate soluble radioactive material is quite low and approximately equal to the scintillation background which has not been subtracted. A low level of formation of N-formyl-C^{14}-met-puromycin is noted even in the absence of the ApUpG messenger, indicating the functional nature of binding of N-formyl-C^{14}-met-tRNA to ribosomes which takes place without additional messenger.

Table 1

Formation of N-formyl-C^{14}-met-puromycin assayed by ethyl acetate extraction*

Addition	Recovered as μμmoles N-formyl-C^{14}-met-puromycin	
	minus puromycin	plus puromycin
None	0.10	0.34
ApUpG	0.12	5.18

*conditions of incubation and assay are given under Methods.

In the presence of the codon, however, 5.18 μμmoles of N-formyl-C^{14}-met-puromycin are formed, approximately 43 times the background value. Results may be expressed as in table 1, indicating the extent of the reaction, or in terms of transfer units, μμmoles of N-formyl-C^{14}-met-puromycin extractable after a ten minute second stage incubation of reaction mixtures as defined above. Transfer units, expressing the linear rate of the reaction, may be used in determining the specific activity of various components which catalyze or are required for the initial peptide bond forming reaction.

The results shown in fig. 2 indicate that the sole radioactive product of the reaction which is extracted by ethyl acetate from aqueous solution at pH 5.5 is actually N-formyl-C^{14}-met-puromycin. In fig. 2A is an electrophoretogram in which the extracted product is shown to migrate with authentic N-formyl-met-puromycin at pH 3.5. Mild acid hydrolysis of

this product yielded the labeled product which is shown in fig. 2B to migrate with authentic met-puromycin, the expected product. In experiments already described (Zamir, Leder, and Elson, 1966), the extracted product has been shown to migrate as a single spot in three chromatographic systems and has been subject to further chemical characterization, confirming its identity as N-formyl-C^{14}-met-puromycin.

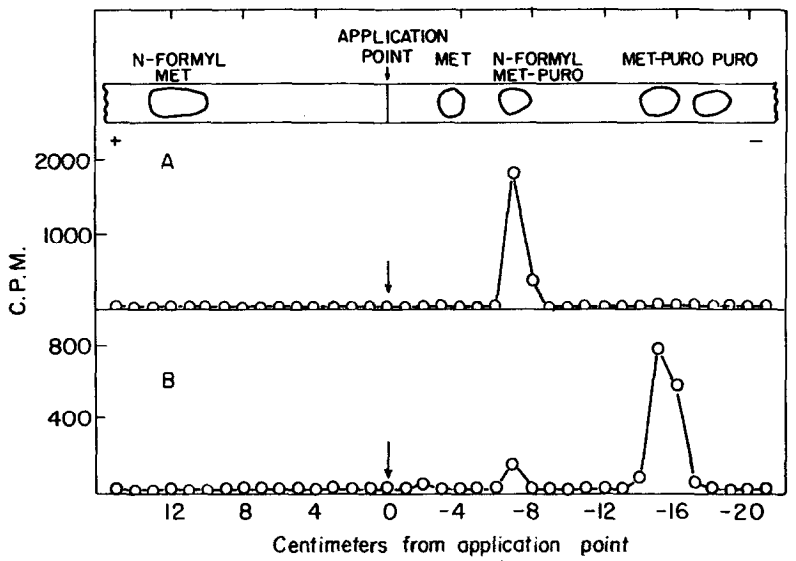

Fig. 2. A. Electrophoretic mobility at pH 3.5 of the ethyl acetate extractable compound from N-formyl-C^{14}-met-puromycin forming reaction mixture. B. Product shown in Fig. 2A after acid hydrolysis. Details of electrophoresis and acid hydrolysis given in the text.

DISCUSSION

The ability to select an isolated event in the sequence of reactions involved in the initiation of protein synthesis, in this case formation of the initial peptide bond, is of considerable value. The procedure described above will conveniently permit identification of those factors involved in formation of the initial peptide bond exclusive of those necessary for polypeptide synthesis. Formation of the penultimate peptide bond involving relative motion of the messenger with respect to the ribosome may also be studied. This may be done through the use of hexanucleotide messengers specifying formyl-methionine at the 5' terminal codon position and a second labeled amino acid in the penultimate position, resulting in the formation of compounds of the following type; N-formyl-met-C^{14}-aminoacyl-puromycin. Further, this assay will permit a detailed investigation of the chemical requirements of the substrates involved in the

initiation process. Preliminary information on certain of these points has recently been described (Zamir, Leder and Elson, 1966) and reports of further studies are in preparation (Leder and Bursztyn, 1966b).

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REFERENCES

Bray, A.G., Anal. Biochem. 1 (279) 1960.
Bretscher, M. and K.A. Marcker, Nature 211 (380) 1966.
Clark, B.F.C. and K.A. Marcker, J. Mol. Biol., 17 (394) 1966.
Kellogg, D., B. Doctor, J. Loebel and M. Nirenberg, Proc. Natl. Acad. Sci. Wash., 55 (912) 1966.
Leder, P. and H. Bursztyn, Proc. Natl. Acad. Sci. Wash., 1966a in press.
Leder, P. and H. Bursztyn, 1966b, manuscript in preparation.
Nathans, D., Proc. Natl. Acad. Sci., 51 (585) 1964.
Nirenberg, M.W. and P. Leder, Science, 145 (1399) 1964.
Sheehan, J.C. and D.H. Yang, J. Amer. Chem. Soc., 80 (1154) 1958.
Smith, J.D., R.R. Traut, G.M. Blackburn and R.E. Monro, J. Mol. Biol. 13 (617) 1965.
Zahn, H. and E. Rexroth, Z. Analyt. Chem. 148 (181) 1955.
Zamir, A., P. Leder and D. Elson, Proc. Natl. Acad. Sci. Wash. 1966, in press.