A RAPID ASSAY FOR POLYPEPTIDE CHAIN TERMINATION

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An assay has been developed for examining the biochemical mechanism of polypeptide chain termination.[1] This assay employs RNA from the bacteriophage R17 in which a glutamine codon (CAG), early in the coat protein gene, has mutated to the chain terminating codon UAG.[2] The RNA from this mutant directs the cell-free synthesis of a small NH_2-terminal coat protein fragment, N-formyl-met-ala-ser-aspn-phe-thr, which, as a result of the UAG codon, is prematurely released from the protein synthesizing machinery.[3,4] The beauty of this system for studying polypeptide chain termination is that one can easily distinguish (and measure) the coat protein fragment still attached to the peptidyl sRNA (which is TCA precipitable) from the released peptide (which is TCA soluble). To make the assay really functional a rapid quantitative method of detecting the coat protein fragment was required. In this communication, such a method is reported.

Methods

(a) Cell-free amino acid incorporating experiments: A one ml reaction mixture contained: 300 μg mRNA, either from the wild type or mutant (amB_2) R17; 250 μl preincubated S-30; 750 μg sRNA; 7 μmole $MgCl_2$; 30 μmole NH_4Cl; 3 μmole ATP; 0.2 μmole GTP; 5 μmole PEP; 20 μg pyruvate kinase; 10 μmole glutathione; 50 μmole Tris, pH 7.8; and 40 mμmole of each amino acid. After 12 min of incubation at 34°C, aliquots of the reaction mixture were assayed

for the released coat protein fragment.

(b) Electrophoresis: Thin layer electrophoresis was done in a Brinkman migration chamber using 500 mm cellulose plates. The samples were applied to the plate in 0.2 x 2 cm strips. Electrophoresis was done at 0°C for 3.25 hours at 38 volts/cm. The electrophoresis buffer contained, per liter, 6.25 ml of acetic acid and 7.0 ml of pyridine. On completion of the electrophoresis, the plates were dried and sprayed with Neatan to facilitate handling. The electropherograms were cut into 0.5 x 4 cm strips and assayed for radioactivity using toluene-liqui-flour (Pilot Chemical Inc., Mass.) on a Packard scintillation counter.

(c) The peptide assay: To a 100 µl reaction mixture (see Section (a)), 400 µl of cold 5% TCA was added and the precipitate collected by centrifugation. The TCA phase, containing the released coat protein fragment, was transferred to a second 12 ml conical tube. The peptide was extracted from the TCA by vigorous mixing with one ml of organic solvent (0.7 ml of cresol + 0.3 ml ethyl-acetate). The phases were separated by centrifugation for 5 min at 25°C (or the phases invert). A 750 µl aliquot of the organic phase was removed. The peptide was then chased into an aqueous phase by mixing with 200 µl of H_2O + 2.0 ml of ether. After removal of the organic phase the aqueous phase was given a second ether wash. Care must be taken to remove the ether before applying the sample to a 0.6 x 2.5 cm Dowex 50 column. (The column was prepared from an acid-washed medicine dropper plugged with glass wool.) The coat protein fragment was eluted from the column with 1.8 ml of H_2O directly into scintillation vials. 20 ml of Brays solution[5] was added and the sample assayed for radioactivity on a Packard scintillation counter. One can readily handle 50 assays a day.

Results

A method was sought to purify sufficiently the coat protein fragment to allow quantitation by direct radioactive analysis. This required exploiting three properties of this peptide: 1) it is TCA soluble; 2) at acid pH's it

is a neutral and hydrophobic peptide; and 3) at neutral pH's it is an acidic peptide. The progress of the peptide assay can be followed by analyzing each step by electrophoresis (see fig. 1).

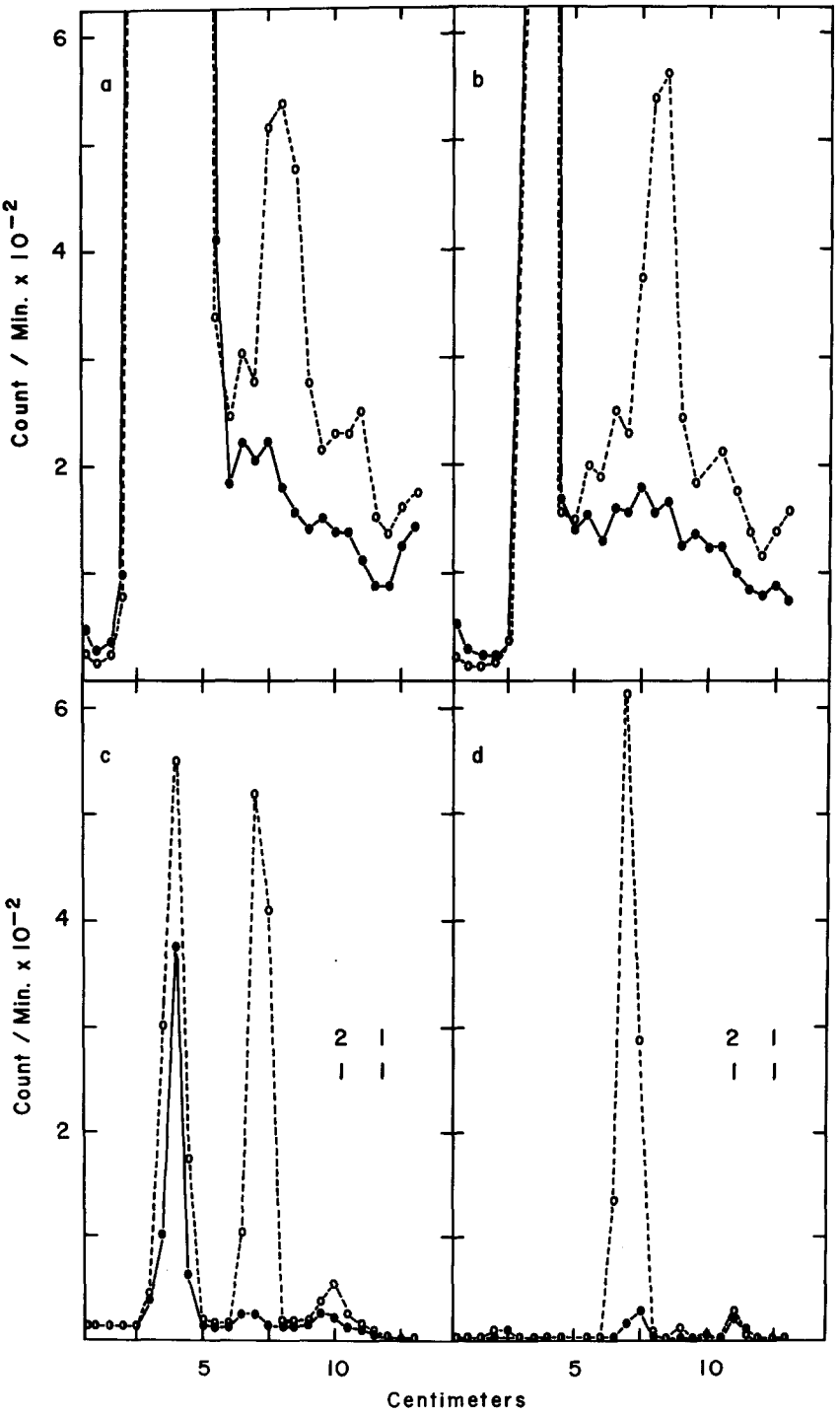

Fig. 1 - Electrophoretic analysis of each step in the purification of the released coat protein fragment, N-formyl-met-ala-C^{14} ser-aspn-phe-C^{14}-thr. (a) the total reaction mixture; (b) the TCA soluble product; (c) the C^{14}-labeled product susceptible to organic extraction; (d) the final product eluted from a Dowex 50 column. Two experiments are superimposed in each frame: 1) cell-free amino acid incorporation directed by the mutant R17 RNA (o--o--o); 2) cell-free amino acid incorporation mediated by wild type R17 RNA, which should not direct the synthesis of the released coat protein fragment (•-•-•). Each experiment represents the analysis of a 100 μl aliquot from a common reaction mixture. The conditions of synthesis and analysis of the reaction mixture are fully discussed in Methods. The peptide standards, N-formyl-met-ala and N-formyl-met-ala-ser, were run with the electropherograms shown in figs. c and d. Their position is indicated by the numbers 1 and 2 respectively. These non-radioactive standards were detected by the platinic iodide reagent.[6]

Figure 1a records an electropherogram of the starting material, the total cell-free amino acid incorporating system. Two experiments are superimposed. The open circles designate the in vitro protein product synthesized under the direction of the mutant R17 RNA. The closed circles designate the cell-free product mediated by wild type R17 RNA, which should not direct the synthesis of the released coat protein fragment. The large number of radioactive counts near the origin (3.5 cm) include: free labeled amino acids (C^{14}-serine and C^{14}-threonine); in vitro synthesized polypeptides still bound to the ribosomes or peptidyl sRNA; and released phage protein which does not migrate toward the anode. The released coat protein fragment, N-formyl-met-ala-C^{14}-ser-aspn-phe-C^{14}-thr, can be readily detected in the 7.5-8.5 cm region by comparison with the C^{14}-labeled peptides synthesized under the direction of wild type R17 RNA.

The smaller radioactive peak in the 10-11 cm region (o--o--o) has been identified by coelectrophoresis and cochromatography with a standard (N-formyl-met-ala-ser), as the tripeptide N-formyl-met-ala-C^{14}-ser. This tripeptide arose from proteolytic hydrolysis of the hexapeptide, N-formyl-met-ala-C^{14}-ser-aspn-phe-C^{14}-thr. Such proteolytic hydrolysis can be maintained at a minimum by limiting the period of incubation in the original reaction mixture. This is possible because proteolysis is much slower than the synthesis and the release of the coat protein fragment. In experiments where proteoly-

sis cannot be tolerated, for example when measuring the specific release of the completed polypeptide chain, an additional trick is to radioactively label only the last amino acid in the polypeptide chain. As we will see, the peptide assay selects for peptides with a blocked amino group, for example peptides starting with formylmethionine. Therefore, proteolytic hydrolysis anywhere in the polypeptide chain would result in the loss of the radioactive label.

Figure 1b records an electropherogram of the TCA soluble product which includes C^{14}-labeled amino acids (near the origin) and the released coat protein fragment. Having removed the released coat protein fragment from the reaction mixture in the TCA phase, the peptide still bound to the peptidyl sRNA can, at this stage, be recovered from the TCA precipitate by base hydrolysis of the peptidyl-sRNA bond. Purification of the artificially released coat protein fragment would proceed in parallel with the released peptide.

At acid pH's the coat protein fragment is a neutral, hydrophobic peptide susceptible to organic extraction. Figure 1c records an electropherogram of the radioactive product soluble in the organic phase. A large pro-

Table I

Purification Step	Total C^{14}-counts	% Recovery
a.	1800	---
b.	1650	92
c.	1330	74
d.	1300	72

The values recorded in Table I are the sum of the C^{14}-counts (beyond 6 cm) of the product synthesized under the direction of the mutant RNA minus the product synthesized under the direction of the wild type R17 RNA. The values obtained for figs. 1c and 1d were normalized to account for only 3/4 of the sample being analyzed (see Methods).

portion, though not all, of the C^{14}-serine and C^{14}-threonine remained behind in the TCA phase.

The final purification of the coat protein fragment was obtained by eluting the peptide from a Dowex 50 column (see fig. 1d).

An estimate of the recovery of C^{14}-labeled coat protein fragment at each purification step is shown in Table I.

Conclusion

In conclusion, a rapid quantitative assay for the coat protein fragment, N-formyl-met-ala-ser-aspn-phe-thr, has been described. This assay is applicable for detecting both the released peptide (which is TCA soluble) and the peptide still bound to the peptidyl sRNA (which is TCA precipitable). The assay has proved to be extremely reproducible ($\pm$ 2%) and was recently employed to demonstrate the existence of a new enzymatic factor required for polypeptide chain termination.[1]

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