THE DISTRIBUTION OF ^{14}C-PROLINE PEPTIDES SYNTHESIZED *IN VITRO* DIRECTED BY POLYCYTIDYLIC ACID; THE EFFECT OF CHLORAMPHENICOL

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1. Introduction

Chloramphenicol (CAP) is a known protein synthesis inhibitor in bacteria [1–3] which has been shown to inhibit the puromycin reaction [4–8]. This inhibition suggests that CAP's action occurs at or before the peptide bond forming step. The inhibitory action of CAP on *in vitro* amino acid incorporation directed by synthetic polynucleotides is dependent upon the particular messenger used; both polyuridylic acid-directed phenylalanine incorporation and polyadenylic acid-directed lysine incorporation are relatively resistant to the action of CAP, whereas polycytidylic acid-directed proline incorporation is quite sensitive to the antibiotic [9–11].

This communication reports the analysis of the ^{14}C-proline peptides synthesized *in vitro* directed by polycytidylic acid and the effect of CAP on the peptide distribution.

The peptide distribution of the ^{14}C-proline peptides synthesized *in vitro* directed by polycytidylic acid was determined by a peptide mapping technique. It was found that the major products were very small peptides and large polypeptides of proline. Also it was determined that these large polypeptides were the only species precipitable by 15% trichloroacetic acid. CAP was found to inhibit the formation of all the peptides. The results suggest that CAP inhibits peptide chain initiation in the polycytidylic acid system.

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2. Materials and methods

Ribosomes were prepared and purified from *E. coli* Q-13 (General Biochemicals) by DEAE-cellulose chromatography according to the method of Stanley and Wahba [12] with minor modifications. The S-100 fraction was prepared according to the method of Nirenberg [13] and concentrated by $(NH_4)_2SO_4$ precipitation (76% saturation) followed by dialysis prior to use. Incorporation was performed at 37° for 40 minutes in a volume of one milliliter containing the following ingredients: 100 mM NH_4HCO_3; 16 mM $Mg(Ac)_2$; 1.0 μM ATP; 0.03 μM GTP; 1.0 μM PEP; 0.3 E.U. pyruvate kinase; 6 mM mercaptoethanol; 0.4 mg stripped tRNA; 1.0 μCi ^{14}C-proline (225 mCi/mM); 100 μl S-100; 1.25 mg ribosomes; 100 μg polycytidylic acid; and 25 μg chloramphenicol when added. After incorporation was terminated bu chilling in an ice bath, the samples were heated for two minutes at 95°. The coagulated protein was removed by filtration and the filtrates containing the proline peptides were lyophilized to dryness. The samples were then treated with 0.5 ml of 0.2 N KOH and 800 μg of carrier peptides, obtained from a partial acid hydrolysate of L-polyproline. The samples were lyophilized to dryness and suspended in a small volume of 1% Triton X-100 in water and spotted on a sheet of Whatman 3 MM paper (40 × 25 cm). Peptide separation was performed by electrophoresis in 4% formic acid at 37.5 V/cm for 3.5 hr in one dimension followed by chromatography in the second dimension in butanol: acetic acid: water, 5:1:4 (v:v:v), upper phase. Carrier peptides were detected by reaction with ninhydrin

and radioactive peptides detected by autoradiography. The radioactive peptides were analyzed quantitatively by cutting the regions from the map that were indicated to contain peptides as determined by either ninhydrin reaction or autoradiography and counting them in a liquid scintillation spectrometer using a toluene based scintillation fluid.

ATP, GTP, PEP, and pyruvate kinase were obtained from Calbiochem; tRNA from Mann Scientific; ^{14}C-proline from Amersham/Searle, polycytidylic acid and L-polyproline from Miles Chemicals; standard proline peptides from Cyclo Chemicals; and chloramphenicol was a gift of the Parke-Davis Co.

3. Results and discussion

Fig. 1 shows a peptide map of the ^{14}C-proline peptides synthesized *in vitro* directed by polycytidylic acid. The identities of the areas designated 1, 2, 3 and 4 were determined from the electrophoretic and chromatographic properties of standards of proline, diproline, triproline, and tetraproline respectively. Areas 5 and 6 are assumed to correspond to the penta- and hexaproline respectively due to their relative migration in the electrophoretic field. Areas 7 and 14 are peptide fractions assumed to correspond to peptides of increasing molecular weight with respect to increasing fraction number and were removed, as shown, in 2-cm fractions. The designation DKP stands for the diketopiperazine of L-prolylproline which was isolated from a map and identified by cochromatography with a standard of L-prolylproline diketopiperazine. The designation PP stands for polyproline; this species is found at the origin of the map. This material, when isolated and subjected to partial acid hydrolysis, gave a two-dimensional map similar to that given by carrier peptides produced from the partial acid hydrolysis of L-polyproline. The 15% trichloroacetic acid precipitable peptides were analyzed by the mapping technique and it was discovered that these precipitable peptides remained at the origin of the map as does the peptide fraction designated PP.

The synthesis of all proline peptides is inhibited in

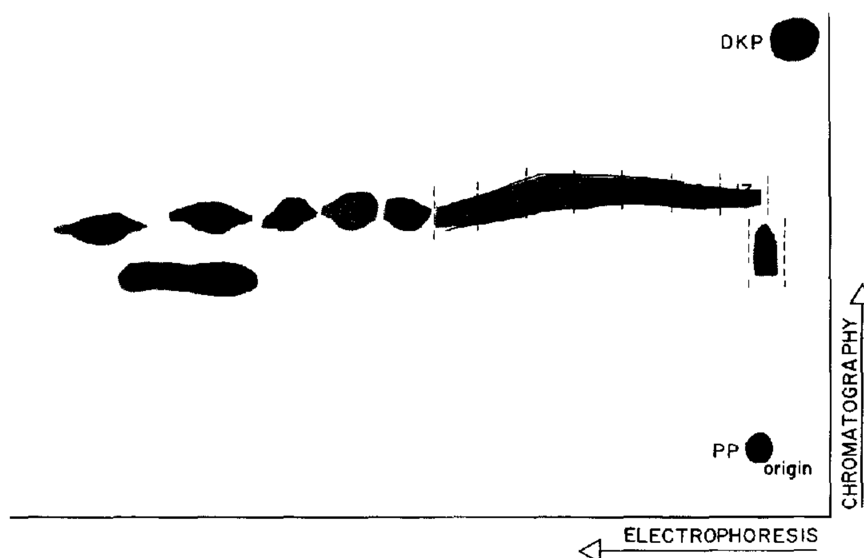

Fig. 1. A peptide map of the ^{14}C-proline peptides synthesized *in vitro* directed by polycytidylic acid. Solid lines indicate the ninhydrin positive carrier peptides; shaded areas indicate radioactivity determined by autoradiography; dashed lines indicate fractions taken of unresolved peptides. Area 1 corresponds to proline, areas 2, 3, 4, 5 and 6 correspond to di-, tri-, tetra-, penta-, and hexaproline respectively, areas 7 to 14 correspond to unresolved peptide fractions. The area designated DKP corresponds to diketopiperazine of L-prolylproline, and the area PP is designated polyproline.

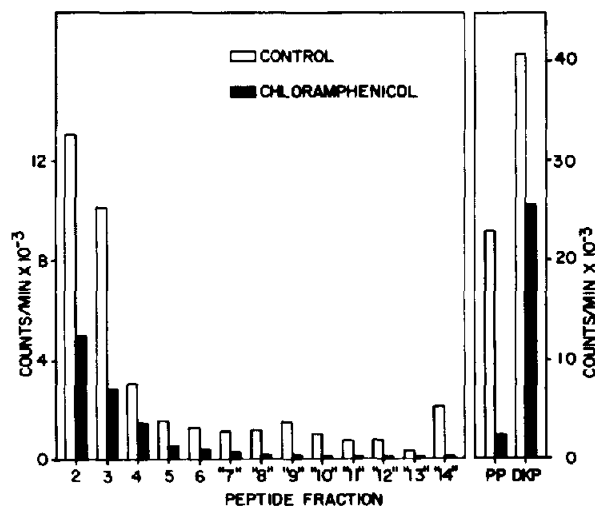

Fig. 2. The distribution of ^{14}C-proline peptides synthesized *in vitro* and the effect of 25 μg/ml chloramphenicol. These data have been corrected for the background obtained from an incorporation sample incubated without polycytidylic acid and analyzed as the other samples. The peptide fraction designations are explained in the legend to fig. 1 and in the text.

the presence of CAP as shown in fig. 2. This is in contrast to the effect of CAP on the *in vitro* synthesis of ^{14}C-lysine peptides directed by polyadenylic acid [14] and of ^{14}C-phenylalanine peptides directed by polyuridylic acid [15]. In the polyadenylic acid system, CAP inhibits the formation of peptides larger than the tripeptide and permitted the formation of di- and tripeptides in amounts greater than observed in the control. A similar situation is observed in the polyuridylic acid system in which the formation of the dipeptide is allowed in a greater amount than observed in the control. In the polycytidylic system the synthesis of the di- and tripeptides is inhibited by the antibiotic but to a lesser extent than the large peptides. It should be noted that the inhibition of proline peptide synthesis occurs at much lower concentrations of CAP than those inhibitory for the formation of lysine and phenylalanine peptides. At 25 μg/ml of CAP total proline peptide synthesis is inhibited 60%; at 400 μg/ml CAP total lysine peptide synthesis is inhibited 55% [14]; and at 180 μg/ml of the antibiotic total phenylalanine peptide synthesis is not inhibited at all [15].

These results suggest that in the polycytidylic acid system CAP is inhibiting an initiation step and not peptide chain extension as it appears to act in the polyadenylic acid and polyuridylic acid systems. This explanation might account for the observed differential sensitivity of the three synthetic messengers. Also it may indicate that CAP acts by two different mechanisms, i.e. inhibition of peptide chain initiation at low concentrations and inhibition of peptide extension at high concentrations. The effect of CAP on the three synthetic messenger systems suggests that the polyadenylic acid and polyuridylic acid systems are initiated in some manner which is not affected by CAP since the formation of dilysine and diphenylalanine is not inhibited by CAP even at high concentrations [14–16]. In two recent articles by Pestka the mechanism of CAP action is stated to be that of inhibiting the binding of the aminoacyl-terminus of aminoacyl-tRNA to the ribosome [16, 17]. Such a mechanism is consistant with the observations of CAP's effect on the polycytidylic acid system reported here. Also inhibition of the synthesis of all the proline peptides is observed at CAP concentrations as low as 1 μg/ml [18]

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