THE ROLE OF PYRROLIDONE CARBOXYLIC ACID IN THE INITIATION OF IMMUNOGLOBULIN PEPTIDE CHAINS

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SUMMARY - Incorporation studies with intact mouse myeloma cells have been carried out to establish whether the N-terminal residue of nascent immunoglobulin heavy chains has a free NH_2-group or is pyrrolidone carboxylic acid (PCA). The myeloma cells incorporate actively glutamine, whereas they do not incorporate PCA. The lack of incorporation of PCA is probably due to impermeability of the cell membrane. The presence of PCA in the terminal position of growing peptide chains in membrane-bound polyribosomes of myeloma cells incubated with [^{14}C] glutamine has been assayed by two different methods. Digestion with pronase, followed by chromatography of the digest on Dowex-50, failed to provide unequivocal results. Direct reaction of the polyribosome-bound peptide chains with fluorodinitrobenzene, followed by hydrolysis and separation of the dinitrophenyl-amino acids, showed that some chains have an NH_2-terminal glutamic acid or glutamine. These results are discussed in relation to the hypothesis that PCA can serve as the initiator amino acid in the biosynthesis of immunoglobulin peptide chains.

The presence of pyrrolidone carboxylic acid (PCA) in the N-terminal position of several proteins (1) has suggested the possibility that it acts as the initiator amino acid in the biosynthesis of these proteins. The use of an amino acid with a blocked amino group for chain initiation is supported by the discovery that in procaryotic organisms initiation is carried out by formyl-methionyl-tRNA (2). However, no evidence has been obtained so far for the presence of an initiator aminoacyl-tRNA with a blocked α-amino group in the cytoplasmic sap of eucaryotic organisms (3-5). Such an aminoacyl-tRNA appears to be present in the chloroplast (6) or mitochondrial (7) system of protein synthesis only.

The present experiments were undertaken with the objective of establishing whether PCA is incorporated as such into nascent polypeptide chains, possibly by being charged to a specific initiator tRNA. Previous reports have suggested that PCA-tRNA can be formed in a mammalian system (8) and that the papaya latex enzyme glutamine cyclotransferase catalyzes the formation of

PCA-tRNA from E. coli glutaminyl-tRNA (9).

A mouse myeloma tumor has been used in the present experiments. This tumor synthesizes and secretes a single immunoglobulin, the heavy chains of which have PCA in N-terminal position (P. Knopf, personal communication). This tumor provides a suitable material for the study of the incorporation of PCA into proteins, specially since it is easy to isolate from myeloma cells membrane-bound polyribosomes, which are involved in the synthesis of immunoglobulins (10).

MATERIALS AND METHODS - Inbred BALB/C mice were obtained from Bar Harbor, Maine. Tumors were produced in mice by subcutaneous injection of suspensions of cells of the Adj-PC-5 myeloma according to Potter (11). The animals were sacrificed after 12 to 14 days and the tumor excised and washed with chilled Eagle minimal basal medium (without L-glutamine) obtained from Microbiological Associates Inc., Bethesda, Md. This medium supplemented with 5% fetal calf serum was used in all the incubations. The tumors were teased and the cells gently dispersed according to Kuff and Roberts (12). The cell suspension was filtered through cheesecloth and the cells sedimented by low-speed centrifugation.

One volume of packed tumor cells was resuspended in 20 volumes of medium and incubated at 37°C after the addition of labeled amino acids (New England Nuclear). After the incubation the cells were centrifuged at 3,000 rpm for 10 min, resuspended in 4 volumes of Medium A containing 0.88 M sucrose and homogenized according to Vassalli (10). The homogenate was centrifuged 10 min at 10,000 rpm in a Servall R2B centrifuge and the precipitate discarded. The supernatant was centrifuged for 45 min at 60,000 g and the supernatant discarded.

The microsomal pellet was resuspended in 3 ml of Medium A containing 0.2 M sucrose by gentle homogenization; 5% sodium deoxycholate was then added to 0.5% concentration. The microsomes were then centrifuged at 10,000 rpm for 10 min to eliminate some insoluble material and the clear

supernatant was layered on 7 ml of Medium A containing 1.3 M sucrose. The tubes were centrifuged for 3 hours at 150,000 g to obtain free polyribosomes and a ribosome-free supernatant called "microsomal extract". The polyribosome pellet was dissolved in 3 ml of 8 M urea containing 1% $NaHCO_3$ and reacted with 0.1 ml of fluorodinitrobenzene (FDNB) for 90 min at room temperature under constant agitation as suggested by Frankel-Conrat et al. (13). At the end of the reaction 0.1 ml of ammonia were added; after 15 min 6 ml of water and 3 ml of 25% trichloroacetic acid were added. After one hour the protein was filtered on a Whatman GF/2 glass filter, washed with alcohol/ether (1/1) and ether, and dried. The dry filter was inserted in an hydrolysis tube containing 6N HCl and heated for 16 hours at 110°C under vacuum. Dinitrophenyl-amino acids were extracted from the hydrolyzate with ether (13). The ether extract was concentrated and analyzed by thin layer chromatography after the addition of appropriate carrier DNP-amino acids (14). The spots on the chromatograms were cut out and counted in a liquid scintillation counter after the addition of 0.1 ml of acetic acid and 5 ml of toluene containing 0.8% butyl-PBD (Beckman).

RESULTS - A preliminary experiment was carried out to establish the most effective labeling procedure for the myeloma cells (Fig. 1). It was found that PCA did not penetrate into the cells and that glutamine was more efficiently utilized by these cells than glutamic acid. Henceforth glutamine was used in all subsequent incubations.

Since glutamine incorporation proceeded rapidly, a 3 minute incubation was thought sufficient to saturate the pool of polyribosome-bound nascent chains with this amino acid. To prepare uniformly labeled immunoglobulin the cells were instead incubated for two hours and the extracellular medium used as a source of labeled protein. This was purified by fractional precipitation with ammonium sulphate between 30 and 55% saturation. The protein thus obtained was dialyzed against distilled water and liophylized.

In preliminary experiments an attempt was made at measuring the ter-

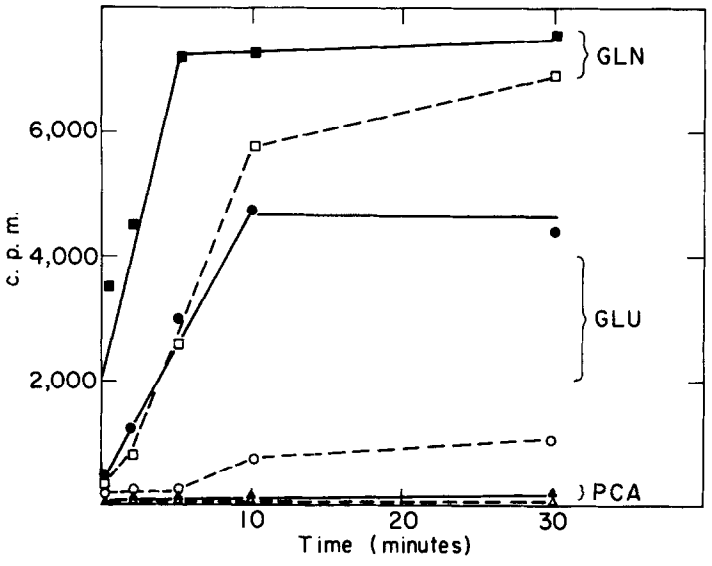

Fig. 1. Rate of entry into myeloma cells and incorporation of glutamine (GLN) glutamic acid (GLU) and pyrrolidone carboxylic acid (PCA). 0.5 ml of packed myeloma cells were incubated 5 min in 10 ml of medium at 37° C; 5 μC of [^{14}C] glutamine, glutamic acid or PCA were then added. 1 ml aliquots were withdrawn at the times indicated and poured into ice cold saline. One aliquot was plated directly on Whatmann GF/2 glass filters and washed with saline, whereas another aliquot was precipitated with 5% trichloroacetic acid and heated for 5 min at 90° C before plating. The solid lines indicate the total label present in cells, while the dotted lines indicate the label present in hot trichloroacetic acid precipitable material.

minal labeled PCA vs. the internal glutamic acid plus glutamine. Control experiments showed that glutamine was not converted into other amino acids besides glutamic acid during a two hour incubation. The labeled immunoglobulin secreted during this incubation was hydrolyzed and analyzed by high voltage ionophoresis (15). The amino acids were identified by the ninhydrin reaction and eluted into scintillation vials with N acetic acid. No significant amounts of counts were found in spots other than glutamic acid.

The terminal vs. internal label introduced into immunoglobulin and nascent chains by the incubation with [^{14}C] glutamine was estimated by digesting the secreted protein and the polyribosome-bound chains with pronase (16). The digest was passed through a Dowex-50 column (1 x 10 cm) equilibrated with water and an aliquot of the digest and of the fraction not adsorbed by

TABLE I

Measurement of terminal PCA labeling vs. internal labeling

Sample	Incubation	cpm	cpm from Dowex-50	% terminal labeling
Immunoglobulin	2 hours	31,500	740	2.35
Polyribosome-bound chains	5 min	9,100	151	1.65

0.5 ml of packed myeloma cells were incubated in 10 ml of medium with 10 μC of [^{14}C] glutamine. The secreted immunoglobulin and the polyribosome-bound chains were isolated as described under Methods and digested with pronase (16). The digest was passed through a Dowex-50 column and the amount of label not adsorbed to the column measured. It has been shown previously (16) that the N-terminal tripeptide or dipeptide obtained by pronase digestion of immunoglobulin heavy chains is not absorbed by Dowex-50, since it does not have a free NH_2-group. The amount of label passed through Dowex-50 provided an estimate of the terminal labeling in PCA, whereas the total label present in each preparation provided an estimate of the internal plus terminal labeling

Dowex-50 counted. The result of this experiment (Table I) indicated that relatively more PCA is present in terminal position in the secreted immunoglobulin than in polyribosome-bound peptide chains, whereas one would expect the ratio of terminal over internal labeling of growing chains to be twice that of completed chains. However, several explanations may account for this observation. The membrane-bound polyribosomes of myeloma cells may be involved in the synthesis of other proteins besides immunoglobulins or the excess light chains synthesized in these cells (17) may contribute more to the internal than to terminal labeling.

A direct attempt to establish whether the N-terminal amino acid of growing heavy chains has a free NH_2-group or is PCA, was carried out by reacting polyribosome-bound chains with FDNB. Polyribosomes, microsomal extract and secreted immunoglobulin were isolated from cells incubated with [^{14}C] glutamine (see Table II). These fractions were reacted with FDNB and hydrolyzed. The label present in the ether extract of the hydrolyzate was measured and the DNP-amino acids separated by two-dimensional thin layer

TABLE II

Measurement of NH_2-terminal labeling vs. internal labeling

Sample	Incubation	cpm	cpm in ether phase	% terminal labeling
Polyribosome-bound chains	3 min	31,500	189	0.60
Microsomal extract	30 min	57,600	220	0.38
Immunoglobulin secreted	2 hours	90,500	320	0.35

0.5 ml of packed myeloma cells were incubated with 20 µC of [^{14}C] glutamine. The microsomal extract and the immunoglobulin secreted were precipitated with 5% trichloroacetic acid, washed with alcohol/ether (1/1) and ether, and dried. The samples were reacted with FDNB, hydrolyzed and extracted with ether as indicated under Methods.

chromatography. Approximately 60% of the cpm of the ether extract were found in the DNP-glutamic spot and no label was found in any other DNP-amino acid. Losses during the concentration of the ether extract and the chromatography step, plus the different efficiency in counting may explain this recovery (Table II). The results obtained should thus be considered more from a qualitative point of view than from a quantitative one, in consideration also of the losses of DNP-glutamic acid which presumably occurred during the hydrolysis (13) and which have not been corrected for.

DNP-glutamic acid can be isolated from polyribosome-bound chains and to a lesser extent from protein present in the microsomal extract and secreted immunoglobulin (Table II). This seems to indicate that at least some of the N-terminal amino acid in polyribosome-bound chains is glutamic acid or more likely glutamine. Whereas several proteins with N-terminal glutamic acid have been found, no protein with N-terminal glutamine has yet been reported to my knowledge.

There is no direct evidence that the DNP-glutamic comes from growing

heavy chains of immunoglobulin. The majority of the membrane-bound polyribosomes in these cells are involved in the synthesis of immunoglobulins (17) but it cannot be excluded that a different protein with N-terminal glutamic is also synthesized on these polyribosomes. The small amount of DNP-glutamic acid obtained by reacting the secreted immunoglobulin with FDNB seems to indicate that a small proportion of the heavy chains of this protein has a free NH_2-terminal group.

Attempts to detect PCA-tRNA in the cytoplasm of myeloma cells incubated with [^{14}C] glutamine have been unsuccessful (18). Moreover, attempts to charge highly purified [^{14}C] PCA to tRNA extracted from myeloma cells have also been unsuccessful (18). Thus, in consideration of the failure to observe a species of tRNA which can be charged with PCA in myeloma cells and of the finding that some polyribosome-bound chains have an available NH_2-terminal, I favour the hypothesis that PCA is formed by cyclization of an NH_2-terminal glutamine residue. Cyclization may occur already on polyribosomes or possibly during or after secretion. Spontaneous cyclization of glutamine in N-terminal position is known to occur during the isolation of peptides (19). Moreover, an enzyme has been reported which catalyzes cyclization of N-terminal glutamine in peptides to PCA (20).

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