Glutamic Acid as a Precursor to N-Terminal Pyroglutamic Acid in Mouse Plasmacytoma Protein

(protein synthesis/initiation/immunoglobulins/pyrrolidone carboxylic acid)

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ABSTRACT Cell suspensions derived from a mouse plasmacytoma (RPC-20) that secretes an immunoglobulin light chain containing N-terminal pyroglutamic acid can synthesize protein in vitro. Chromatographic examination of an enzymatic digest of protein labeled with glutamic acid shows only labeled glutamic acid and pyroglutamic acid; hydrolysis of protein from cells labeled with glutamine, however, yields substantial amounts of glutamic acid in addition to glutamine and pyroglutamic acid. The absence of glutamine synthetase and presence of glutamine synthetase in plasmacytoma homogenates is consistent with these findings. These data indicate that N-terminal pyroglutamic acid can be derived from glutamic acid without prior conversion of glutamic acid to glutamine. Since free or bound forms of glutamine cyclize nonezymatically to pyroglutamate with ease, while glutamic acid does not, the data suggest that N-terminal pyroglutamic acid formation from glutamic acid is enzymatic rather than spontaneous.

Pyroglutamic acid (pyrrolidone carboxylic acid; pGlu) is found as the N-terminal amino acid in various proteins and polypeptides. It has been demonstrated in both immunoglobulin light and heavy chains (1), fibrinogen (2), and collagen (3), as well as in kinins and venoms (4). Although pGlu has a blocked amino group and a structure about its α-carbon atom similar to that of N-formylmethionine, there is no convincing evidence implicating it in the initiation process. Since pGlu cannot be acetylated directly to transfer RNA (5, 6), attempts have been made to demonstrate a mechanism for pGlu formation in proteins similar to the formylation of methionine on fMet-tRNA (7, 8). Preliminary studies demonstrating that pGlu is formed from glutaminyl-tRNA (9) or from N-terminal glutamine (10) are available, but the spontaneous cyclization of glutamine to pGlu (11) makes the interpretation of these data difficult. This tendency of glutamine and N-terminal glutaminyl peptides to form pGlu under mild conditions has introduced some reservations as to whether pGlu is present in native protein or is derived from the spontaneous cyclization of glutamine (11).

We report here experiments that identify the precursor of N-terminal pGlu in plasmacytoma protein. A preliminary report of this work has been published (12).

METHODS

Preparation of Plasmacytoma Cell Suspensions. Tumors (RPC-20) were transplanted subcutaneously in female, inbred BALB/c mice. Freshly excised tumors were washed and minced in a solution of 5% Puck’s D, (13) saline containing 2% sucrose and 0.1% glucose. The cell suspension (w/v = 1/5) was filtered through a Mylar screen (mesh size 10.5 mm), sedimented by low-speed centrifugation then washed twice in chilled Dulbecco’s phosphate-buffered saline (pH 7.2). Cells were resuspended in Eagle’s minimum essential medium (MEM) (14), without glutamine (cells from 1 g of tumor/10 ml of media) and the suspension was equilibrated with 5% CO₂-95% air. Incubation at 37°C was initiated by the addition of radioactive amino acids.

Enzyme Assays. Preparation of homogenates and reaction mixtures for the radiochemical assay of glutamine synthetase (EC 6.3.1.2) were as described by Lund (15). Glutaminase (EC 3.5.1.2) activity was measured by a modification of the glutamine synthetase assay, with radioactive glutamine as the substrate. The loss of radioactivity in the effluent from a Dowex-1 column was a measure of the enzyme activity.

RESULTS

Amino acid incorporation by cell suspensions of mouse plasmacytoma

Cell suspensions of mouse plasmacytoma, when incubated in Eagle’s minimum essential medium, actively incorporate radioactive amino acids into protein; maximum incorporation is reached at about 30 min (Fig. 1). Substantial incorporation was observed for glutamine, glutamic acid, leucine, histidine, and methionine. The great difference in the extent of incorporation of glutamine compared to leucine and glutamic acid, as shown on the semilogarithmic plot, probably is due to differences in uptake rates or pool sizes in the cells. The data of Fig. 2 support this idea. As shown in the bar graph, the addition of unlabeled glutamine to cell suspensions stimulated the incorporation of glutamic acid and leucine. This stimulation was not observed for the other amino acids tested. This is in keeping with the greater requirement for glutamine than for other amino acids for the growth of L cells and HeLa cells in tissue culture (16).

Identification of the precursor of pGlu in plasmacytoma protein

A cell suspension was prepared in minimal essential medium minus glutamine. One 10-ml incubation contained 54 nmol (100 μCi) of [³H]glutamic acid, in the presence of an equimolar amount of cold glutamine; another incubation contained 54 nmol of [¹⁴C]glutamine (10 μCi), in the presence of an equimolar amount of cold glutamic acid. After 90 min of incubation at 37°C, the cell suspensions were frozen and thawed to break the cells, concentrated by lyophilization,
dialyzed extensively against H₂O, and again lyophilized. The lyophilized material was then suspended in 2 ml of 0.05 M Tris-HCl (pH 8.0) and digested with Pronase (Calbiochem, 100 mg, 4500 proteolytic units) for 24 hr at 37°C. Any remaining insoluble material was removed by centrifugation.

A preliminary analysis of protein hydrolyzates from each cell incubation was made on Dowex-50 columns. pGlu, which has a blocked amino group, is not retained on the negatively-charged resin and is found in the effluent. About 10% of the radioactivity derived from protein labeled with either glutamine or glutamic acid was found in the effluent (Table 1). The mobility on high-voltage electrophoresis [0.05 M potassium formate (pH 4.5)] of the radioactive material in column effluents was identical with that of standard pGlu.

The protein hydrolyzates described above were also examined by ascending paper chromatography in phenol-water 88:12 to identify the amino acids labeled by [³H]glutamic acid or [¹⁴C]glutamine. Chromatography of the hydrolyzate from cells labeled with glutamic acid (Fig. 3, upper panel) demonstrated that only glutamic acid and pGlu were labeled. There was no evidence of conversion of glutamic acid to glutamine. In contrast, an examination of the protein hydrolyzate from cells labeled with glutamine (Fig. 3, lower panel) showed substantial amounts of labeled glutamic acid and glutamine, in addition to pGlu. We suggest therefore that the pGlu in protein from glutamate-labeled cells is derived from glutamic acid without the intervention of a glutamine intermediate.

Enzyme activities in plasmacytoma and liver homogenates

The above hypothesis is supported by a survey of glutamine synthetase and glutaminase activity in plasmacytoma cell extracts. The results of Fig. 4 (upper panel) indicate that glutamine synthetase activity was not detectable in plasmacytoma homogenates, whereas substantial activity was seen in mouse liver. On the other hand, a very active glutaminase activity (Fig. 4, lower panel) was observed in plasmacytoma

![Graph](image)

**Fig. 1.** Plasmacytoma cell suspensions in medium without glutamine and leucine were prepared. Each of three 10-m1 incubation mixtures were supplemented with 54 nmol of glutamic acid, glutamine, and leucine. One incubation contained [¹⁴C]-glutamine (10 μCi), another [¹⁴C]glutamic acid (13.7 μCi), and the third [³H]leucine (100 μCi). The mixtures were incubated at 37°C in screw-capped tubes. 1-ml samples were removed at the time indicated, precipitated by the addition of 1 ml of 10% Cl₂COOCH₃, and then heated in a boiling-water bath for 5 min. The precipitates were trapped on glass-fiber filters and washed with 5% Cl₂COOCH₃. Radioactivity on the filters was determined by scintillation counting with 10 ml of triton-toluene-Liquifluor 6:12:1.

**Table 1.** Analysis of protein digests on Dowex-50 columns

<table>
<thead>
<tr>
<th>Label</th>
<th>Applied</th>
<th>In effluent</th>
<th>% In effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>[¹⁴C]Glutamine</td>
<td>6770</td>
<td>735</td>
<td>10.8</td>
</tr>
<tr>
<td>[³H]Glutamic acid</td>
<td>4855</td>
<td>413</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Protein labeled with [¹⁴C]glutamine or [³H]glutamic acid was hydrolyzed with Pronase. 0.5-ml samples of the hydrolyzate were applied to columns (0.5 × 4 cm) of Dowex-50W × 2 (hydrogen form, 200-400 mesh). The columns were washed with 1.0 ml of H₂O. The combined effluent and washes were counted with 10 ml of Triton-toluene-Liquifluor (New England Nuclear Co.) counting fluid.
extracts, while liver extracts showed undetectable activities of this enzyme.

**DISCUSSION**

This study has focused attention on two interrelated questions: whether glutamic acid or glutamine is the more direct precursor of pGlu in protein, and whether N-terminal pGlu formation in immunoglobulins is a biological or artifactual process. A metabolic distinction between glutamic acid and glutamine is difficult in many tissues because of the enzymatic interconversion of the two amino acids catalyzed by glutamine synthetase or glutaminase. Thus, while this study might have been complicated in other tissues, the unique properties of the plasmacytoma extracts allowed us to obtain interpretable results. The absence of glutamine synthetase (Fig. 4) prevents glutamine formation from glutamic acid (Fig. 3, upper panel), although the glutaminase present does not suppress glutamic acid formation from glutamine. These data are consistent with previous observations that mammalian cells in culture have a glutamine requirement that cannot be replaced by glutamic acid (16).

The artifactual formation of pGlu occurs primarily with glutamine, and not with glutamic acid. Table 2 emphasizes this distinction between glutamic acid and glutamine. Neither glutamic acid nor glutamyl-tRNA formed significant amounts of pGlu after incubation at pH 8.0 and 37°C. On the other hand, about 8% of the added glutamine was recovered as pGlu after 18 hr under these conditions. The nonenzymatic cyclization of glutamine probably explains the finding (Table 1) that pGlu was recovered from hydrolysates of glutamine-labeled protein (10% after 24 hr at pH 8.0). When the α-carboxyl group of glutamine was esterified (glutaminyl-tRNA), the nonenzymatic cyclization was accelerated about 4-fold. This distinction in the chemical stabilities of glutamic acid and glutamine led us to anticipate different possible interpretations of our precursor studies. If we had found that glutamine was the precursor to pGlu, we would be uncertain whether the isolated pGlu had been formed by an enzymatic or nonenzymatic process. Since the data suggested that pGlu was derived from glutamic acid, the interpretation was greatly simplified. We can, therefore, assume that an enzymatic process is necessary for the cyclization of glutamic acid to pGlu.

Fig. 5 outlines two possible models for the conversion of glutamic acid to pGlu. In the first mechanism, analogous to the formation of formylmethionyl-tRNA in bacterial systems, glutamyl-tRNA is cyclized to pyroglutamyl-tRNA. An ATP-dependent phosphorylation of the γ-carboxyl of the

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**Fig. 3.** Ascending paper chromatography in 88% phenol-12% H2O of radioactive protein hydrolyzate from cells labeled with [3H]glutamic acid (upper panel) or [14C]glutamine (lower panel). The relative mobilities of glutamic acid, glutamine, and pGlu in this solvent are indicated in the upper panel.

**Fig. 4.** Homogenates from liver and plasmacytomas were prepared in 4 volumes of 0.05 M Tris-HCl, (pH 7.5)-5 mM EDTA-0.9% NaCl. Cellular debris was removed by centrifugation at 10,000 × g, and the supernatant was used for both the glutamine synthetase and glutaminase assay. Incubation mixtures for the glutamine synthetase assay (top) were as described by Lund (15); glutaminase was assayed (bottom) in 0.05 M Tris-HCl (pH 8.5). Reaction mixtures from both assays were equilibrated to 37°C and reactions were started by addition of the crude homogenate. Samples were withdrawn at various times and pipetted into 0.25 ml of cold 95% ethanol; the precipitate was removed by centrifugation and the supernatants were assayed on Dowex-50 columns.

**Fig. 5.** Mechanisms for pGlu formation from glutamic acid.
enzymatic cyclization, precipitated The 0.1 M column to a applied 5 ^Ci/mol, glutamic in completely 0.15 phenolpyruvate; mixtures for [14C]glutamine (187 B Escherichia coli Table 1. X 8.6 10' Tris-HCl, pH 8.0, containing acid, 5.5 X 10^6 cpm; [14C]glutamine, 5.5 X 10^6 cpm; [14C]glutaminyl-tRNA, 8.6 X 10^5 cpm. 0.05-mI Aliquots of the incubations were diluted to 1.0 ml with H2O and passed through Dowex-50 columns as in Table 1. Aminoacyl-tRNA preparations were made by incubation of E. coli B aminoacyl-tRNA synthetase preparations. Incubation mixtures for aminoclaylation contained: 50 mM potassium cacodylate buffer (pH 7.2); 10 mM MgCl2; 5 mM ATP; 2 mM phosphoenolpyruvate; 50 A_m/ml E. coli B tRNA (Schwarz Bio-Research); 0.1 mg (43 units)/ml pyruvate kinase (Sigma); about 0.15 mg/ml aminoacyl-tRNA synthetase preparation, sufficient to completely acylate the tRNA with glutamine or glutamic acid in 15 min. For acylation with glutamine, the mixture included [14C]glutamine (187 Ci/mol), 1.25 uCi/ml. For acylation with glutamic acid, the incubations contained [3H]glutamic acid (15 Ci/mol), 5 uCi/ml. After incubation for 15 min, the mixture was applied to a column (1.3 X 4 cm) of DEAE-cellulose, and the column was washed with about 25 ml of 0.3 M NaCl containing 0.1 M sodium acetate (pH 4.5)-10 mM MgCl2-1 mM EDTA. The bound tRNA was then eluted with 10 ml of 1 M NaCl containing the same additions as the 0.3 M NaCl buffer. tRNA was precipitated by the addition of 3 volumes of cold ethanol. The precipitate was dissolved in 0.05 M Tris-HCl (pH 8.0).

esterified glutamic acid, followed by either a spontaneous or enzymatic cyclization, would have a precedent in similar resctions (17). Pyroglutamyl-tRNA, as an initiator tRNA, might respond to specific codons and initiation factors. The second suggested mechanism involves the post-translational conversion of protein-bound N-terminal glutamic acid to N-terminal pGlu. This mechanism does not dictate that the protein be initiated by glutamic acid. pGlu might be formed in conjunction with the proteolytic cleavage of an adjacent amino acid or peptide. Further experiments designed to discriminate between these alternative mechanisms are in progress.

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