The Essentiality of Decyclization of Pyrrolidonecarboxylic Acid Prior to Utilization in Protein Biosynthesis

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SUMMARY

Pyrrolidonecarboxylic acid (PCA) is not incorporated directly into a protein polypeptide chain but can be converted into a utilizable form by a decyclase factor. The detection of radioactivity in newly synthesized protein produced by cells incubated in medium containing PCA-14C was indicative of the presence of decyclase. The biosynthetic demonstration of PCA decylase in a human plasmacytoma producing a Bence Jones protein with a cyclized glutaminyl amino-terminal residue contrasted to the absence of demonstrable decyclase in a murine plasmacytoma, which was also producing a Bence Jones protein with a cyclized glutaminyl amino terminus. These results indicate a lack of correlation between the presence of PCA decyclase and the occurrence of cyclized glutaminyl as the amino-terminal residue of a polypeptide chain.

INTRODUCTION

The amino-terminal residue of the heavy chains of most immunoglobulins as well as of most λ light polypeptide chains is characterized by an absence of a free α-amino nitrogen group (4) and is designated Gip. Cyclization of glutamine or glutamic acid can be effected chemically and results in the formation of the compound PCA. The existence of cyclase factors for glutamine and glutamic acid have also been demonstrated (2, 3, 9, 11). Previous studies indicated that the amino-terminal residue Gip was formed by the cyclization of glutamine (1) and that PCA was not incorporated directly into the polypeptide chain (1, 7, 10) or into tRNA (10, 13). PCA can be converted into a utilizable form by an enzyme capable of decyclizing the PCA molecule (13).

To investigate whether a direct correlation exists between the presence or absence of demonstrable decyclase or cyclase and the specific amino-terminal residue Gip, we performed comparative biosynthetic studies with cells derived from a human plasmacytoma that produced a Bence Jones protein with a Gip amino terminus and with cells from 3 different murine plasmacytomas: Adj-PC9, which synthesized a Bence Jones protein with Gip in the amino-terminal position; MOPC-46B, which produced a Bence Jones protein with an aspartyl amino-terminal residue; and Adj-PC5, which produced a Η immunoglobulin with a Gip amino-terminal residue on the heavy chain and an aspartyl amino-terminal residue on the light polypeptide chain (4). The significance and resulting implications of finding decyclase activity only in the human plasmacytoma are discussed.

MATERIALS AND METHODS

Reagents. Omnifluor scintillant, L-glutamic acid-3H (specific activity, 2 Ci/mmole), and L-glutamine-14C (specific activity, 94.7 mCi/mmole) were purchased from New England Nuclear, Boston, Mass. Chromatographic analyses (see below) revealed that 5% of the radioactivity of the glutamic acid and 15% of the radioactivity of the glutamine represented labeled material, which corresponded to the RF value of PCA. Pronase (Grade B) was obtained from Calbiochem, Los Angeles, Calif., and Nuclear-Chicago solubilizer solvent was purchased from Nuclear-Chicago, Des Plaines, Ill. L-PCA was purchased from the Aldrich Chemical Co., Inc., Milwaukee, Wis. L-Lysine-14C (specific activity, 231 mCi/mmole) and L-isoleucine-14C (specific activity, 308 mCi/mmole) were obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y. Bio-Gel P-100 polyacrylamide (100 to 200 mesh) and AG 50W-X2 (H+) cation-exchange resin were purchased from Bio-Rad Laboratories, Richmond, Calif.

Preparation of PCA-14C. Labeled PCA was prepared from glutamine-14C (13) as follows: a glass ampul containing 0.9 ml of glutamine-14C (100 μCi/ml) and 0.1 ml of 1 M sodium phosphate buffer, pH 6.5, was evacuated, sealed, and maintained in a 100° water bath for 2 hr. Radiochromatographic assay of an aliquot of the labeled material (Chart 1) revealed that approximately 98% of the radioactivity was associated with a compound that had the same RF value as PCA. The PCA-14C preparation was then diluted 10-fold with water and stored at −20°.

Pronase Digestion. Five to 10 mg of lyophilized Bence Jones protein were dissolved in 1 ml of water, and the pH was

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4The abbreviations used are: Gip, cyclized glutaminyl or glutamyl; PCA, pyrrolidonecarboxylic acid.

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adjusted to 8.2 with 0.001 M NaOH. Pronase was added at an enzyme to protein ratio of 1:10 (w/w), and the mixture was incubated at 37° for 3.5 hr. The pH was maintained at 8.2 during incubation by the addition of 0.001 M NaOH.

Chromatographic Procedures. Descending chromatography was performed on 2.5- x 50-cm strips of Whatman No. 3MM paper in 1-butanol:acetic acid:water (2:1:1, v/v/v) for 18 hr at room temperature. Starch iodide (12) and ninhydrin stains were used in the identification of PCA, glutamine, and glutamic acid. Glutamate and glutamic acid react with both stains, whereas PCA reacts only with the starch iodide stain. The Rf values of PCA, glutamine, and glutamic acid were 0.72, 0.36, and 0.48, respectively.

A 1.5- x 10-cm column of the AG 50W-X2(H+) cation-exchange resin was used in the chromatographic separation of peptides produced by Pronase digestion. The Pronase-digested material was applied to the column of resin, which had been washed thoroughly with deionized, double-distilled water. The uncharged peptides in the digested material were not bound to the resin. Water was added to the resin column until no starch iodide-positive material was detected in the eluate. The bound peptides were eluted with 1 M NH₃OH; the collection of the eluent was terminated when no ninhydrin-positive material was detected. The water eluate and the ammonium hydroxide eluate were both lyophilized.

Chemical Analyses. Protein concentration was measured by a modification of the Folin-Ciocalteu method (8). The dansylation procedure was performed according to the method of Gray (6). Peptides were prepared for amino acid analyses (Spinco Model 120 analyzer) by hydrolysis in 6 N HCl for 20 hr.

Radioactivity Analyses. Samples were prepared for assay by solubilization in 1 ml of Nuclear-Chicago solubilizer solvent, and the resulting mixture was added to counting vials containing 15 ml of Omnifluor-toluene solution. Chromatograms were prepared for assay of radioactivity by cutting of the paper into 1- x 2.5-cm strips, which were placed in counting vials containing 15 ml of Omnifluor-toluene. The radioactivity was assayed in a Packard Tri-Carb liquid scintillation spectrometer.

Biosynthetic Studies. A 3-cm section of a human plasmacytoma was minced into 1-cm fragments. Five to 8 fragments were placed in 12- x 125-mm glass, screw-top culture tubes and were washed 3 times with 10 ml of Hanks' balanced salt solution. The tumor fragments were suspended in 2 ml of modified McCoy's 5A medium (Grand Island Biological Co., Grand Island, N. Y.) to which had been added 4 µCi of either PCA-14C, glutamine-14C, or glutamic acid-3H plus 10 mg of ovalbumin crystallized 3 times (Nutritional Biochemicals Corp., Cleveland, Ohio). This medium, as supplied, lacked glutamine, glutamic acid, serum, and antibiotics. Cultures were also established in Medium 320 (kindly supplied by Dr. Richard Asofsky, National Cancer Institute) to which were added both lysine-14C and isoleucine-14C to yield a final concentration of 1 µCi of each labeled amino acid per ml of medium. Medium 320, as supplied, lacked lysine, isoleucine, glutamine, serum, and antibiotics. L-Glutamine (Baltimore Biological Laboratory, Baltimore, Md.) and ovalbumin were added to yield concentrations of 0.3 and 5 mg/ml, respectively.

Duplicate cultures were established for each labeled amino acid. The tubes were placed in a roller drum and incubated at 37° for 23 to 28 hr. The culture fluids were harvested by centrifugation at 230 X g for 10 min at 4°. An aliquot of extracellular fluid from each culture was frozen, and the remaining fluid was dialyzed at 4° in 20/32 Visking tubing against 4 changes of a 200-fold volume of 0.15 M NaCl over a 24-hr period to remove unincorporated radioactive amino acids.

Newly synthesized Bence Jones protein was isolated from each culture fluid as follows: 25 mg of the patient's Bence Jones protein (previously isolated from a specimen of urine by P-100 gel filtration) were added to an aliquot of dialyzed culture fluid, and the resulting sample was applied to a 2.5- x 100-cm P-100 polyacrylamide column and eluted with a Tris-HCl buffer, which was composed of 0.15 M NaCl and 0.05 M Tris-HCl (pH 7.6) and contained 0.001 M EDTA and 0.02% sodium azide. The effluent was monitored at a wavelength of 254 mμ. The fractions that contained Bence Jones protein were pooled; dialyzed against deionized, double-distilled water; lyophilized; and stored at −20°.

The murine plasmacytomas Adj-PC9, MOPC-46B, and Adj-PCS were kindly supplied by Dr. Michael Potter, National Cancer Institute. The tumors were transplanted by s.c. inoculation and maintained in BALB/c female mice (Jackson Memorial Laboratory, Bar Harbor, Maine). When approximately 2 cm in diameter, the tumors were excised and used for transplantation or for biochemical studies. The methods of tissue culture were as described above. Newly synthesized protein was isolated from extracellular culture fluid as follows: 0.35 ml of culture fluid was added to 0.35 ml of carrier protein (4 mg/ml). The protein was precipitated by the addition of 0.70 ml of cold 5% trichloroacetic acid and was harvested by centrifugation at 1000 X g for 10 min at 4°. The precipitate was washed twice with 1 ml of cold 5% trichloroacetic acid and was dried under vacuum. Nonspecific radioactivity was determined by processing a control sample (culture media containing carrier protein and radioactive amino acids but no tumor tissue).

Preparation of pH 5 Extract and Analysis for PCA Decyclase Activity. Extracts (pH 5) from the liver and from the tumor of BALB/c mouse bearing each of the 3 types of plasmacytomas were prepared by the method of Weinstein et al. (15). The assay for PCA decyclase activity was performed as described previously (13). The reaction mixture contained, per 0.2 ml, 3.05 mg of protein of the pH 5 extract, 0.5 µCi of PCA-14C (specific activity, 94.7 mCi/mmmole), 1.0 µmole of MgCl₂, 0.4 µmole of ATP, 4 µg of pyruvate kinase, 0.5 µmole of phosphoenolpyruvate, and 10.0 µmoles of Tris-HCl, pH 7.4. After 30 min of incubation at 37°, the reaction mixture was frozen. The percentage of PCA-14C converted to glutamic acid-14C was determined by radiochromatography.

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RESULTS

Chemical Studies on the Human Bence Jones Protein. The human Bence Jones protein was classified immunochemically as a protein of type L.6 The resistance of the Bence Jones protein to dansylation suggested that the amino-terminal amino acid residue lacked a free α-amino nitrogen group and that the protein, like most λ light polypeptide chains, had Glp as amino terminus (4). Confirmation that the amino-terminal residue of the polypeptide chain was Glp was obtained as follows: the Bence Jones protein was treated with Pronase, and the digest was applied to a cation-exchange resin equilibrated in water. Peptides with a blocked α-amino group were not bound to this resin and were readily eluted by water; the bound peptides were eluted by ammonium hydroxide. Paper chromatographic analysis of the water eluate revealed a distinct starch iodide-positive, ninhydrin-negative spot at the origin; free PCA was not detected. The amino acid analysis of the peptide obtained in the water eluate revealed approximately equimolar amounts of glutamic acid and serine. Therefore, it was concluded that the amino-terminal amino acid residue of the protein was Glp and that the sequence of the amino-terminal dipeptide was Glp-serine.

Bioisynthetic Studies with Tumor Cells from a Human Plasmacytoma. Newly synthesized, i.e., labeled, Bence Jones protein was identified and isolated from extracellular culture fluids obtained after incubation of tumor cells in medium containing either radioactive PCA, glutamine, or glutamic acid. Gel filtration of the culture fluid and subsequent assay for radioactivity revealed labeled material that eluted in the same column volume as carrier (unlabeled) Bence Jones protein. Similar assays were performed on culture fluids obtained from incubation of tumor cells in medium containing both lysine-14C and isoleucine-14C; concordantly, radioactivity was detected in the eluate containing Bence Jones protein.

To determine the contribution of the amino-terminal residue to the total radioactivity of the Bence Jones protein molecule, we treated each isolated sample of labeled protein with Pronase and chromatographically separated the amino-terminal peptide from the remainder of the protein digest. The radioactivity present in the amino-terminal peptide of Bence Jones protein synthesized by tumor cells incubated in the presence of labeled PCA, glutamine, or glutamic acid represented 1.5, 1.6, and 0.7%, respectively, of the total radioactivity of the intact polypeptide chain. The amino-terminal peptide of the protein synthesized by cells incubated in medium containing labeled lysine-14C and isoleucine-14C; concordantly, radioactivity was detected in the eluate containing Bence Jones protein.

Isoleucine-14C Total radioactivity (dpm) NH3-terminal radioactivity % of total

<table>
<thead>
<tr>
<th>Radioactive amino acid</th>
<th>Total radioactivity</th>
<th>NH3-terminal radioactivity</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine-14C</td>
<td>3575</td>
<td>54</td>
<td>1.5</td>
</tr>
<tr>
<td>Lysine-14C</td>
<td>4171</td>
<td>68</td>
<td>1.6</td>
</tr>
<tr>
<td>Glutamic acid-3H</td>
<td>4833</td>
<td>35</td>
<td>0.7</td>
</tr>
<tr>
<td>PCA-14C</td>
<td>3830</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Radioactivity contained in the water eluate plus radioactivity contained in the ammonium hydroxide eluate.
b Radioactivity contained in the water eluate.

ing amino-terminal peptide was performed. A starch iodide-positive, ninhydrin-negative spot was localized at the origin of the chromatogram; the PCA-14C-derived radioactivity was localized to this site. No peak of radioactivity was detected at the site where free PCA or glutamine would be expected. The radioactivity of the amino-terminal peptide of the protein synthesized in the presence of labeled glutamine or glutamic acid was also located at the origin.

Bioisynthetic Studies with Tumor Cells from 3 Different Murine Plasmacytomases. The 3 murine plasmacytomases have been associated with the formation of specific types of immunoglobulins and have been characterized as follows: Adj-PC9 tumor cells synthesize a κ-type Bence Jones protein with a Glp amino-terminal residue; MOPC-46B tumor cells synthesize a κ-type Bence Jones protein with an aspartyl amino-terminal residue; and Adj-PC5 tumor cells synthesize a 7S immunoglobulin in which the heavy chains possess a Glp amino terminus and the light chains possess an aspartyl amino terminus (4).

Cells derived from each of the plasmacytomases were incubated for 23 hr in medium that included either labeled PCA, glutamine, or glutamic acid. The incorporation of radioactivity derived from each labeled amino acid into newly synthesized protein was determined by ascertaining the radioactivity contained in trichloroacetic acid-precipitable material in each of the extracellular culture fluids (Table 2). Cells from the murine plasmacytomases, like those from the human plasmacytoma, incorporated labeled glutamic acid and glutamine into the polypeptide chains being synthesized; however, in contrast to the results obtained with cells from the human plasmacytoma, incorporated labeled glutamic acid and glutamine deficient in their capacity to utilize PCA (i.e., to convert PCA to a form suitable for incorporation) in protein synthesis. The production of labeled proteins by murine tumor cells in cultures containing PCA-14C was markedly less than the production of labeled protein by tumor cells in cultures containing either glutamine-14C or glutamic acid-3H. Three separate studies, each yielding results comparable to those presented in Table 2, were performed.
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Table 2
Incorporation of radioactivity derived from labeled amino acids into murine immunoglobulins

The tissue cultures were established as described in "Materials and Methods" with 1 exception being that the cultures with glutamic acid contained 20 μCi of glutamic acid-3H/ml. Trichloroacetic acid precipitates of extracellular culture fluids were prepared and assayed for radioactivity as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Plasmacytoma</th>
<th>Radioactive amino acids</th>
<th>Trichloroacetic acid-precipitable radioactivity&lt;sup&gt;a&lt;/sup&gt; (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adj PC-9</td>
<td>PCA-14C</td>
<td>3,993</td>
</tr>
<tr>
<td></td>
<td>Glutamine-14C</td>
<td>107,963</td>
</tr>
<tr>
<td></td>
<td>Glutamic acid-3H</td>
<td>97,377</td>
</tr>
<tr>
<td></td>
<td>PCA-14C</td>
<td>1,808</td>
</tr>
<tr>
<td></td>
<td>Glutamine-14C</td>
<td>76,094</td>
</tr>
<tr>
<td></td>
<td>Glutamic acid-3H</td>
<td>18,938</td>
</tr>
<tr>
<td>Adj PC-5</td>
<td>PCA-14C</td>
<td>1,563</td>
</tr>
<tr>
<td></td>
<td>Glutamine-14C</td>
<td>123,334</td>
</tr>
<tr>
<td>MOPC-46B</td>
<td>PCA-14C</td>
<td>43,881</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each value represents the average of 2 separate precipitations of 0.35 ml of the same sample minus the nonspecific radioactivity precipitated in an equal volume of the control sample.

Radiochromatographic analyses of the supernatant fluids from cultures containing PCA-14C indicated that less than 10% of the PCA was converted to labeled material that had an R<sub>P</sub> indicative of glutamic acid (Chart 1). Confirmation of the fact that the murine plasmacytomas were deficient in a factor necessary for the conversion of PCA into a utilisable form was obtained through the demonstration that a pH 5 extract prepared from each of the 3 murine plasmacytomas exhibited no PCA decyclase activity, whereas a similarly prepared extract from the liver of the tumor-bearing mice possessed PCA decyclase activity. Graphical representation of the data is presented in Chart 2.

DISCUSSION

The detection of radioactivity derived from PCA-14C in a newly synthesized human λ Bence Jones protein was indicative of the conversion of PCA to glutamine or to glutamic acid, since it had been established previously that PCA was not incorporated into tRNA (10, 13). The results of our biosynthetic studies with 3 murine plasmacytomas demonstrated that PCA was not incorporated directly. Although the human plasmacytoma, like the murine plasmacytomas, was incapable of direct incorporation of PCA, our studies demonstrated the capacity of this tumor to convert PCA into a form that could be utilized for protein synthesis. Radiochromatographic analyses of undialyzed fluid obtained from cultures of human tumor cells incubated with PCA-14C revealed that the major peak of radioactivity was associated with a component with an R<sub>P</sub> value identical with that of the reference glutamic acid (Chart 3). Similar analyses of fluids obtained from cultures incubated with glutamine-14C showed that glutamine was converted to 2 major components: I had an R<sub>P</sub> identical with that of glutamic acid, and the other had an R<sub>P</sub> of 0.25 and was also detected in the culture fluids derived from cells incubated with glutamic acid-3H or with

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Chart 1. Radiochromatographic analysis of extracellular fluid (---) from the culture of Adj-PC9 tumor cells incubated in the presence of PCA-14C. The positions of reference standards glutamine (GLN), glutamic acid (GLU), and PCA are indicated. The radiochromatogram of the PCA-14C preparation (------) is shown for comparison. The numbers along the ordinate are to be divided by the negative exponential.

Although we were unable to identify this component, we can state that it was neither aspartic acid, nor proline, nor α-ketoglutaric acid, which had R<sub>P</sub> values of 0.36, 0.58, and 0.50, respectively.

Nonetheless, the special significance of these studies resides in the demonstration that a human plasmacytoma possessed a factor capable of effecting the conversion of PCA, thereby rendering it available for incorporation into a protein polypeptide chain. Hence, one can predict that the absence of this factor would result in the failure of PCA to be utilized in protein synthesis. Indeed, we found that there was no conversion of PCA in the cultures of murine tumor cells in which incorporation into protein of PCA-derived radioactivity had not occurred. A factor capable of effecting the decyclization of PCA to glutamic acid was demonstrated by Rush and Starr (13) in pH 5 extracts of rabbit liver. Similarly, a pH 5 extract prepared from the liver of BALB/c mice had
PCA decyclase activity (Chart 2). While the identity between the liver-derived decyclase factor and the decyclase factor present in the human plasmacytoma was not established, it was demonstrated that both factors had the capacity to decyclize PCA. Whether the ability to decyclize PCA was unique to the human plasmacytoma or is characteristic of other human plasmacytomas is not known. Nevertheless, the presence of a decyclization factor in murine liver as well as in rabbit liver suggested to us that PCA decyclase is not species specific.

The existence of a cyclase factor whereby glutamine or glutamic acid is converted to PCA has been demonstrated (9, 11, 23). Upon contemplation of the possible role played by the cyclase and decyclase factors in the synthesis of proteins, one may suggest that together the 2 function to form a control mechanism regulating the rate of protein biosynthesis. One may postulate that a constant rate of protein synthesis by plasma cells is maintained by the cyclase-decyclase control mechanism, which functions to maintain a critical ratio between the amount of glutamic acid (and/or glutamine) and the amount of PCA present within the cell. In a protein-producing cell, the incorporation of glutamic acid (and/or glutamine) into the polypeptide chain may occur directly and/or indirectly via PCA decyclization. If a malfunction occurs that involves the cyclase mechanism, one may predict an alteration in the rate of protein synthesis and an accumulation of the decyclase factor. A malfunction in the decyclase mechanism may also result in an alteration in the rate of protein synthesis and in either an accumulation of PCA or an accumulation of the cyclase factor if glutamic acid is incorporated into the polypeptide chain as readily as it becomes available in the cell.

Hence, one may suggest that the marked difference in decyclase activity of the human plasmacytoma and the 3 murine plasmacytomas reflected a specific characteristic of the individual plasma cells. The human plasmacytoma may have been characterized by cells with a deficient cyclase mechanism, whereas the murine plasmacytomas may have represented neoplasms characterized by cells with a deficient decyclase mechanism. More specifically, the demonstrable decyclase activity of the human plasmacytoma would be interpreted as reflecting a cellular system wherein the production of cyclase was greatly reduced and the cyclase that was available was utilized mainly in the cyclization of the amino-terminal residue of the Bence Jones protein polypeptide chain to form Glp. The reduced amount of cyclase affected a reduction or perhaps a total loss in PCA formation, which in turn effected an accumulation of decyclase. Conversely, the murine plasmacytomas appeared to represent a cellular system wherein the production of decyclase was greatly reduced.
plasmacytoma Adj-PC9 was associated with the production of a Bence Jones protein with Glp as the amino-terminal residue, but in contrast to the human plasmacytoma very little decyclase activity was associated with the Adj-PC9 tumor; these facts suggest that an amino-terminal residue of Glp is associated with neither the presence nor the absence of PCA decyclase but is genetically encoded.

REFERENCES

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