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Thermal Condensation of a Mixture of Six Amino Acids

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Amino acids can be converted to peptides by the process of heating if the mixture is dry and if the appropriate proportion of glutamic acid as the dominant monomer is present. Three thermal peptide fractions of the diffusible fraction were isolated in this study. Trifluoracetic acid (TFA) hydrolysis was utilized to open the blocked N-terminal group before performing the dansylation reaction. When the molecular weight of the thermal peptide increased, the proportion of neutral amino acids increased slightly, with a decrease in the concentration of glutamic acid. Quantitative analysis of amino acid composition is also reported.

INTRODUCTION

Thermal condensation of amino acids common to proteins has been discussed by Fox and Harada (*1*, *2*). The results of these experiments have shown that it is possible to prepare peptides thermally by use of sufficient proportions of the acids such as aspartic acid, glutamic acid, or lysine (*3*) in the reaction mixture. Study of the N-terminal residues was begun in order to determine peptide structure and sequence. However, in some preparations the peptide was found to be inactive when it was subjected to the dansylation reaction or the Sanger method and is ninhydrin negative. Recently, it was found that the N-terminus may be blocked when either glutamic acid (*4*) or lysine (*5*) is the N-terminal residue, present in the form of a cyclic lactam in the case of glutamic acid or an acylated ε-NH_2 group in the case of lysine.

Pyroglutamic acid was first described as a derivative of glutamic acid by Menozzi and Appiana (*6*). It was obtained by heating glutamic acid powder alone at temperatures close to the melting point (180–190°C). The conditions under which glutamic acid is transformed to pyroglutamic acid have been studied in detail by a number of investigators (*7*). Melville (*8*) reported that pyroglutamic acid formation from N-terminal glutamine would be expected during lengthy preparations of proteins and peptides, especially at higher temperatures. The presence of pyroglutamic acid in the polypeptide chains presents serious difficulties in determining the amino acid sequence, because it is not possible to determine the N-terminal amino acids or to attempt the sequencing from the N-terminus of the polypeptide.

The purpose in pursuing these studies of thermopolymerization of amino acids was to understand the possible prebiological origin of proteinlike polymers by thermal polymerization of a simple mixture containing only acidic and neutral amino acids. The investigations of Phillips and Melius (*9*) indicated a pyroglutamic (pGlu) residue as the N-terminus of this type of polypeptide. In the present work, an attempt has been

made to open the lactam ring of N-terminal pGlu, so that the N-terminal amino acid could be identified and the Edman sequencing procedure could be applied to determine the amino acid sequence of the polypeptides.

EXPERIMENTAL PROCEDURE

Thermal Copolymerization of Amino Acids

In the preparation of thermal polypeptides, 2.5 g of glutamic acid and 0.5 g each of glycine, alanine, proline, phenylalanine, and leucine were weighed into a 250-ml flask. The flask was heated on an oil bath at 180–190°C for 12 hr under a stream of nitrogen. The vitreous mixture was dissolved in approximately 20 ml of 50% aqueous pyridine solution containing granular particles. After standing overnight, the product was transferred to dialysis tubing. Dialysis was conducted in $1\frac{1}{8}$-in. cellophane tubing against 25% pyridine for 1 week with agitation of the bath by a magnetic stirrer and a change of the 25% pyridine solution every 24 hr. This procedure resulted in a nondiffusible fraction and a diffusible fraction, but only the bulk of polymer formed was diffusible under these conditions.

Isolation of Thermal Polypeptides

The descending chromatograms were run on Whatman 3MM paper for peptide mapping. The developing solvent used was *n*-butanol:acetic acid:water (BAW, 4:1:5, v/v). Chromatograms were run for 15 hr at room temperature. Three purple peptide bands were detected by the procedure of Rydon and Smith (*10*) and are shown in Fig. 1. These peptide-containing bands were eluted by using 10% acetic acid. The

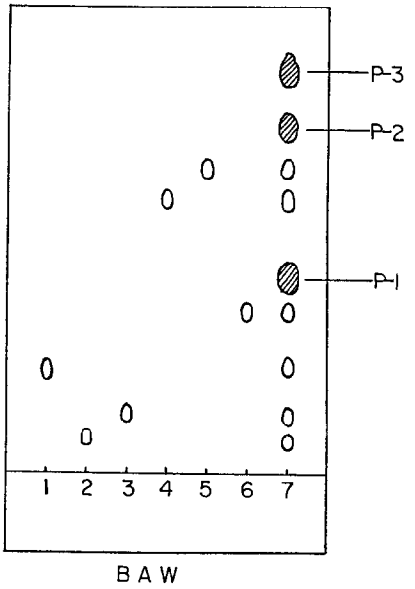

FIG. 1. One-dimensional paper chromatography. Map of the thermal polypeptide in BAW (4:1:5). Open spots, ninhydrin positive; shaded spots, ninhydrin negative, HClO-EtOH–starch–KI positive. (1) Ala; (2) Glu; (3) Gly; (4) Phe; (5) Leu; (6) Pro; (7) thermal polypeptide.

slowest moving peptide was labeled P-1; the fastest moving peptide P-3; and the middle one P-2. Only these three peptide bands were isolated for study. Paper chromatography again was used for further purification of each peptide fraction.

Amino Acid analysis

Qualitative analysis. Peptides were subjected to hydrolysis for 22 hr in 6 *N* HCl at 110°C in evacuated sealed tubes. The identity of amino acids present in the hydrolysates was determined by subjecting the samples to descending paper chromatography in BAW and then spraying with ninhydrin.

Quantitative analysis. Amino acid analysis was carried out by the gas–liquid chromatography procedure of Gehrke (*11–13*) and also on a Beckman Model 121M automatic amino acid analyzer.

End group determination. A 100-μl aliquot of each of the three peptides was treated with 200 μl 1 *N* TFA and placed in a heated sand bath at 80°C for 10 hr under a stream of nitrogen. Removal of TFA was ensured by adding 100 μl of water twice, and evacuating in a vacuum desiccator over P_2O_5 and followed by the dansylation reaction (*14, 15*). The dansyl (DNS)-amino acid was identified by thin-layer chromatography on a silica gel sheet (*16*). Benzene:pyridine:acetic acid (80:20:5, v/v) was used as solvent which was based on that described by Gros (*17*). The solvent front was allowed to migrate around 15–17 cm; the sheet was examined under uv light, and the yellow fluorescent spots were marked. C-terminal amino acids were determined by the hydrazinolysis procedure of Fraenkel-Conrat and Tsung (*18–20*). The hydrazinolysate derivative was allowed to pass through a small column (1 × 6 cm) of Amberlite IRC-50 ion exchanger. The column was then eluted with deionized water, and the effluent was collected in 50 tubes of 2 ml each. The ninhydrin method of Crooke et al. (*21*) was used for amino acid analysis. The resulting solution became purple if it contained free amino acids. The fractions containing amino acids were pooled and concentrated. The C-terminal amino acid freed by hydrazinolysis were identified by dansylation and thin-layer chromatography.

Molecular Weight Estimation

The analytical procedure used was recommended by Weber et al. (*22*). Insulin, cytochrome *c*, lysozyme, myoglobin, alcohol dehydrogenase, serum albumin, and carboxypeptidase A were chosen as standard proteins. Some of the standards showed one dark band and several light bands. The molecular weights of the standards are given in Table 3 (see Results). The molecular weights were calculated by choosing the darkest band. However, for the three peptides only a very clear single band was visible.

RESULTS

Thermal polymerization of amino acids resulted in the formation of glasslike products that varied in color from yellow to amber depending on the heating time. Longer times produced darker products. Yields of 30–40% have been obtained. Qualitative analysis of these three peptides demonstrated that P-1 contained only Ala,

Glu, Gly, and Leu, while P-2 and P-3 contained all six amino acids. The results of paper chromatography were consistent with the analysis by gas chromatography (glc). On glc chromatograms, glutamic acid was converted mainly to the cyclized pyroglutamic acid trimethylsilyl (TMS) derivative, which gave the major peak. The amino acid compositions of thermal peptides were calculated as mole per cent, as shown in Table 1, revealing that glutamic acid predominates either overall or among the individual amino acid fractions.

TABLE 1

AMINO ACID COMPOSITIONS OF POLYMER FRACTIONS

	Fraction (mole %)[a]		
Amino acid	P-1	P-2	P-3
Ala	7.1	24.4	2.8
Gly	26.0	21.7	23.1
Glu	43.4	40.7	37.0
Leu	23.5	4.6	21.0
Phe	—	1.6	6.2
Pro	—	6.9	9.9

[a] The amino acid analyses were done by the gas–liquid chromatography procedure as described in the text.

The DNS-amino acid was very easily identified by thin-layer chromatography since the DNS-amino acids are strongly fluorescent when irradiated with the proper wavelength of uv light. The results are shown in Table 2. The identity of the carboxyl terminal residues were obtained by hydrazinolysis. This procedure results in the C-terminal residue being released as a free amino acid, while the others involved in peptide bonding are converted to hydrazides. The free amino acid was identified by converting it to the dansyl derivative which was then subjected to thin-layer chromatography. Table 2 shows the results of these studies. The notable characteristic of these results is the total absence of glutamic acid in the C-terminal position, even though it was the dominant constituent in the reactant mixture.

TABLE 2

END GROUPS OF POLYMER FRACTIONS

Fraction	N-terminal[a]	C-terminal[a]
Peptide-1	Glu	Gly
Peptide-2	Glu	Ala
Peptide-3	Glu	Leu

[a] The N-terminal residue was determined by treating the peptides with 1 *N* TFA followed by dansylation. The C-terminus was determined by hydrazinolysis.

The mobility of these three peptides and seven standard proteins in 7.5% acrylamide gel for 2.5 hr after sodium dodecyl sulfate (SDS)-gel electrophoresis were calculated as shown in Table 3. By plotting log molecular weight versus mobility, a straight line was obtained, as shown in Fig. 2. The molecular weights of these three peptides were calculated as 5200, 10 000, and 11 500 for P-1, P-2, and P-3, respectively.

TABLE 3

MOLECULAR WEIGHTS AND ELECTROPHORETIC MOBILITIES OF STANDARD PROTEINS AND THERMAL PEPTIDES

Protein	Molecular weight	Mobility[a]
Insulin	6000	1.14
Cytochrome *c*	12 350	0.93
Lysozyme	16 800	0.89
Myoglobin	17 200	0.88
Carboxypeptidase A	34 600	0.73
Alcohol dehydrogenase	41 000	0.62
Albumin (bovine serum)	68 000	0.47
Peptide-1	5200	1.17
Peptide-2	10 000	1.00
Peptide-3	11 500	0.96

[a] The mobilities were estimated from electrophoresis in SDS gels.

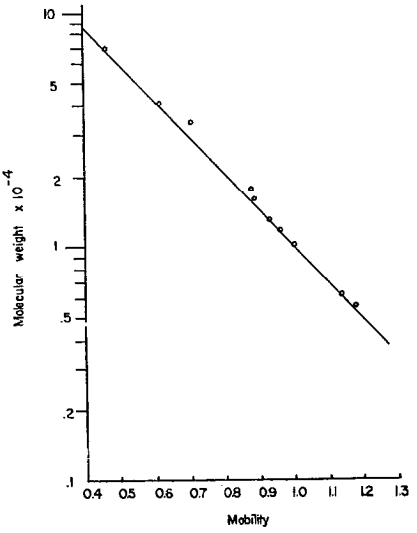

FIG. 2. Comparison of the molecular weights of thermal peptides and the standard proteins listed in Table 3 with their electrophoretic mobilities on standard 7.5% polyacrylamide gels.

DISCUSSION

Thermal condensation of amino acids of course is a dehydration reaction. According to Wilson and Cannan (*23*), in aqueous solution with weak acid or base conditions and high temperature, the formation of lactam is greatly favored. Although glutamic acid

alone was converted by heat virtually entirely to the lactam, it undergoes dehydrations with each of many amino acids to yield linear peptides. It is reasonable to expect that the reaction will occur between the free carboxyl group of the pyroglutamic acid and available amino groups from other amino acids. In addition, pyroglutamic residues provide a directional ordering since the free carboxyl group is the only effective functional group. Therefore, if polymerization went in the opposite direction, it would be necessary to open the lactam ring through transamidation or hydrolysis. It has been pointed out before that many naturally occuring proteins and peptides have been found to have pyroglutamic acid at the N-terminal position (*24–26*). When the protein was subjected to the dansylation reaction or the fluorodinitrobenzene (FDNB) procedure, it obviously did not react at the N-terminus and was ninhydrin negative. It is not possible, due to the absence of a free N-terminal group, to determine the structure and sequence of the isolated thermal polypeptides by the usual methods. In the experiments reported here, all the thermal polypeptides are essentially diffusible, and actually only three peptide fractions were isolated and further purified by paper chromatography. All three of these fractions, P-1, P-2, and P-3, were subjected to the ninhydrin reaction and were found to be ninhydrin negative. Also they did not produce an N-terminal reaction in the dansylation and the Sanger procedures. Thus, it was considered highly probable that the N-terminal residue could only be a pyroglutamic acid. As the fractions P-1, P-2, and P-3 appeared to be homogenous upon paper chromatography, paper electrophoresis, and gel electrophoresis and appeared to have only one predominant C-terminal amino acid, we will subsequently refer to them as peptides. Of course, there is a good possibility of microheterogeneity in these fractions which have similar molecular weights on the basis of the SDS-gel-electrophoresis experiment. In other words, a fraction may consist of more than one peptide with the same amino acid content and end groups but different amino acid sequences. Theoretically, in order to open the lactam ring, a compound must go through a hydrolysis reaction in which water must be present. Therefore, in this experiment the peptides were treated with trifluoroacetic acid in water, and the temperature was increased in order to enhance the reaction rate. Three fluorescent spots were found for the three peptides, after reaction with dansyl chloride. As predicted, pyroglutamic acid was the N-terminus for all these three peptides.

Table 2 indicates the N-terminal and C-terminal groups of the three peptides isolated (P-1, P-2, and P-3) here. It seemed probable that glutamic acid, which is present in highest content in each of the three peptides, would thus have a strong possibility of being the N-terminal group. In P-1, Gly was shown to be present in the next higher amount, in P-2 Ala was present in the next higher amount, and in P-3, Gly again was shown to be in the second highest concentration of amino acids present. Therefore, according to probability from amino acid composition it seemed reasonable to expect that Gly, Ala, and Gly would be the C-terminus of P-1, P-2, and P-3, respectively. For P-1 and P-2, this result was observed. However, for P-3 the C-terminus that was found was not Gly but Leu. Although the content of leucine is comparable to Gly, P-3 contains significant amounts of Phe and Pro which may account in some way for the fact that Leu is the C-terminus of P-3. It may be of interest that P-1 has the same end groups as a thermal peptide identified by Fox and co-workers (*27*).

Comparing the molecular weight with amino acid composition, one can identify two trends. When the molecular weight of the peptide increased, the composition of phenyl-

alanine and proline in the polymer fractions also increased. Also, as the molecular weight increased the proportion of neutral amino acids increased and dicarboxylic acid decreased. Thus it appears that the incorporation of the amino acids is not purely random, and certainly the N-terminus is specifically pGlu in the polymers isolated in this study. The amino acids do exert some directing influences on the amino acid sequences formed. This effect had been suggested earlier by Fox (*28, 29*).

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