CHARACTERIZATION OF AMYLOID FIBRIL PROTEINS FROM MEDULLARY CARCINOMA OF THE THYROID*

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Two different components are detected in human amyloid. One soluble component, the so-called P component, is immunologically homogeneous (1). The other component, the fibril, is more heterogeneous. Fibrils constituted of the variable regions of light immunoglobulin chains are found especially in primary and myeloma/macroglobulinemia-associated amyloidosis, while protein AA type fibrils occur primarily in secondary amyloidosis (for nomenclature see 1).

The occurrence of amyloid is a typical finding in medullary carcinoma of the thyroid (MCT) (2-4). This MCT amyloid exhibits the characteristics of all amyloids such as affinity for Congo red and green birefringence when studied in polarized light. However, there is some evidence for chemical differences from most other amyloids (4, 5). Therefore, we have performed an antigenic and immunchemical analysis of the MCT amyloid and characterized a low molecular weight subunit from purified MCT amyloid fibrils.

Materials and Methods

Preparation of Amyloid Fibrils. Amyloid-rich lymph node metastases of a medullary carcinoma of the thyroid of a 35-yr-old male patient (F. R.) (5) were used as the main source of amyloid. A secondary medullary carcinoma of the thyroid (RO, kindly provided by Doctors R. A. Cook and R. Anders) and rich in amyloid was used for further antigenic comparisons.

For isolation of the P component, the saline washes II-VIII were pooled and used for demonstration of P component (6). Isolation of amyloid fibril proteins followed a modification by Westermark (5) of the method of Pras et al. (7). Since the MCT amyloid was more difficult to dissolve in 0.1 M NaOH than most other amyloids, the solution of degraded amyloid fibrils (DAM) was regularly sonicated.

Antisera. Antisera against amyloid protein AA, protein VAV (AR), void volume (Vo) material, and some light chain amyloid proteins (8, 9), as well as the P component (6), were prepared as previously described. Antisera against MCT amyloid was raised in rabbits using the DAM preparation of MCT amyloid fibril protein FR. Antiserum against a variety of κ- and λ-light chains, Ig classes, and normal human serum were also used. (8).

Gel Filtration. MCT amyloid fibrils from patient F. R. were dissolved in 6 M guanidine HCl – 0.1 M Tris buffer pH 8.0 with 0.1 M dithiothreitol, applied to a 65 × 1.6-cm Sepharose 6B column, equilibrated with 5 M guanidine in H2O, and eluted with the same solution. Suitable fractions were dialyzed exhaustively against distilled water in tubings of slightly reduced porosity and lyophilized.

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Immunodiffusion of antiserum directed against amyloid of a medullary carcinoma (FR) (central well), against DAM preparations of two amyloids of medullary carcinomas (FR in wells 1 and 4 and RO in well 3), and against $A_{\text{MCT}}$ of amyloid FR (well 2). One line of precipitation showing antigenic identity is seen.

**Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis.** SDS electrophoresis was performed using the procedure of Swank and Munkres (10).

**Further Analyses.** The proteins were hydrolyzed and analyzed for amino acids as described before (11). Cyanogen bromide cleavage was performed similar to that of Sletten and Husby and Tager and Steiner (11, 12). Amino acid sequences were determined by a JEOL-JAS-47K Sequence Analyzer (Jeol Company, Tokyo, Japan) and in addition by manual Edman procedure (11). The phenylthiohydantoin amino acids were identified as described before (11).

**Results**

**Immunological Studies.** When DAM preparations of amyloid FR and RO were tested in double immunodiffusion against antiprotein AA, antiprotein $\lambda\lambda\lambda$, or antisera against other known amyloid proteins, no precipitation lines occurred. Neither did anti-$\kappa$, anti-$\lambda$, or antihuman normal serum give any lines of precipitation. In contrast, DAM FR tested with anti-DAM FR absorbed with pooled normal human serum gave one line of precipitation fusing with the precipitation lines obtained with the major low molecular weight subunit material of DAM FR (see below) and with the second MCT amyloid, DAM RO (Fig. 1).

Absorption of the anti-DAM FR antiserum with pooled normal human serum did not diminish the activity of the antiserum. However, some human sera, including such from patients with MCT, had the ability to completely abolish the lines of precipitation.

When anti-amyloid FR antiserum was tested with DAM preparations of 15 systemic amyloids of primary and secondary types and of 5 pancreatic islet amyloids obtained from different diabetic patients, no lines of precipitation occurred.

When P-component antiserum was tested against the saline wash material of amyloid FR, a line of precipitation occurred which was identical with the line of the saline wash material of a control systemic amyloid and with the line of purified P component.
Further Characterization of the MCT Amyloid Protein. Gel filtration on Sepharose 6B of the amyloid material MCT FR yielded a major $V_o$ peak and a smaller second peak called $A_{MCT}$ (Fig. 2). SDS polyacrylamide gel electrophoresis of $A_{MCT}$ material gave only one band corresponding to a mol wt of about 6,000 daltons, and this band represented the major component of the total amyloid material. Amino acid analysis of the amyloid fibrils, as well as gel filtration fractions 18-26 ($V_{o1}$), fractions 27-34 ($V_{o2}$), and fractions 46-51 ($A_{MCT}$) (Fig. 2), showed a rather similar overall amino acid composition for the amyloid fibrils and the $A_{MCT}$ protein, whereas the $V_o$ material was slightly different (Table I). This is in agreement with the results of SDS electrophoresis that the main protein of the MCT amyloid fibrils is the protein $A_{MCT}$. The amino acid composition of the protein $A_{MCT}$ was slightly different from calcitonin (Table I), but all amino acids in calcitonin, except for threonine and phenylalanine fitted into the $A_{MCT}$ protein molecules. The molecular weight of $A_{MCT}$ calculated from the amino acid composition (about 5,700) fitted well with that determined by SDS polyacrylamide gel electrophoresis, and indicated about 53 amino acid residues of protein $A_{MCT}$ (Table I).

N-terminal sequence analysis was performed with both the manual procedure and with the protein program for the automatic sequence analyzer. No N-terminal amino acid could be detected, and when the analyses were continued several steps, no amino acid derivatives were obtained.

Cyanogen bromide cleavage was then carried out. In as much as protein $A_{MCT}$ contained only one residue of methionine, the cleavage product was lyophilized and applied to the sequence analyzer. The peptide program was used, and the sequence was elucidated for 11 residues that should correspond to the N terminus of the second cyanogen bromide peptide. They were: Leu-Gly-Thr-Tyr-(Thr)-Glx-Asx-Phe-Asn-Lys-Phe-. This sequence corresponds to residues 9-19 in human calcitonin which are: Leu-Gly-Thr-Tyr-Thr-Gln-Asp-Phe-Asn-Lys-Phe (13). The first cyanogen bromide fragment corresponding to the N-terminal-blocked fragment was analyzed in SDS polyacrylamide gel electrophoresis, which gave a mol wt of about 3,200 daltons. The residual C-terminal part of the second
### Table I

Amino Acid Composition of Amyloid Fractions Obtained from Medullary Carcinoma of Thyroid Compared with Calcitonin

| Residues per 100 | Residues per molecule |
|------------------|-----------------------|
| VoE | AMCT | AMCT | AMCT | Calcitonin (ref. 13) |
| Asp | 10.4 | 8.80 | 8.49 | 9.52 | 4.74 (5) | 3 |
| Thr | 5.45 | 4.12 | 3.41 | 5.50 | 2.78 (3) | 5 |
| Ser | 7.15 | 6.74 | 5.59 | 7.63 | 3.80 (4) | 1 |
| Glu | 12.3 | 11.3 | 10.1 | 13.8 | 6.88 (7) | 2 |
| Pro | 5.7 | 8.1 | 8.9 | 6.3 | 3.16 (3) | 2 |
| Gly | 10.0 | 21.4 | 26.7 | 12.2 | 6.06 (6) | 4 |
| 1/2Cys | 1.6 | 1.0 | 0.9 | 1.6 | 0.8* (2) | 2 |
| Val | 5.15 | 3.87 | 3.51 | 4.23 | 2.11 (2) | 1 |
| Met | 1.1 | 0.7 | 0.6 | 2.11 | 1.05 (1) | 1 |
| Ile | 3.31 | 2.93 | 2.52 | 2.11 | 1.05 (1) | 1 |
| Leu | 8.24 | 6.49 | 5.14 | 7.93 | 3.96 (4) | 2 |
| Tyr | 4.91 | 2.94 | 3.0‡ | 0.72 | 0.31 (1) | 1 |
| Phe | 4.8 | 3.2 | 3.2 | 3.0 | 1.50 (2) | 3 |
| His | 1.72 | 1.08 | 0.90 | 2.04 | 1.02 (1) | 1 |
| Lys | 5.06 | 4.49 | 3.83 | 5.55 | 2.78 (3) | 1 |
| Arg | 5.46 | 4.45 | 5.05 | 6.34 | 3.16 (3) | 0 |

| Number of residues | 53 |
| Molecular weight | 5,680 |

* Measured as cysteic acid.
† Hydrolyzed without vacuum.

Cyanogen bromide fragment remaining after 15 Edman degradation steps was not detected in the SDS gel, probably because it was too small (mol wt about 800).

**Discussion**

The present finding definitely supports the view that the AMCT protein of MCT is unique and differs from other known amyloid proteins. The antiserum to protein AMCT did not cross-react with any other type of amyloid so far observed, and antisera to other amyloid preparations did not cross-react with DAM preparations of MCT amyloid or with its AMCT protein. It was also shown that protein AA and light chain variable fragments, which are present in many different amyloids (1), are absent in amyloid of MCT. The MCT amyloid occurs only in the vicinity of the tumor cells and seems to be a product of the epithelial cells (2, 3). This amyloid resembles in some respects the amyloid of the islets of Langerhans (14), but they do not seem to be identical.

The AMCT protein is the major protein in the MCT amyloid fibrils. Although the elution profile from the gel filtration shows a relatively small peak of the AMCT protein, this is in agreement with the amino acid analysis and the ultraviolet spectra which revealed a low content of aromatic amino acids. N-terminal analysis of the AMCT protein gave no free N terminal, but Edman degradation of the cyanogen bromide-treated material gave an amino acid sequence corresponding to residues 9–19 in calcitonin. Furthermore, since the mol wt of the AMCT protein was found to be about 5,700 and thus considerably larger than calcitonin, and the amino acid composition was similar, but with distinct differences from calcitonin, there is evidence that the AMCT protein...
represents a prohormone for calcitonin or a fragment of a precursor. The different amounts of threonine and phenylalanine residues found for AMcv and calcitonin may represent genetic variation or be due to a pathological calcitonin. It is not unlikely that hormone-related proteins are involved in the formation of amyloid fibrils also in other endocrine glands than the thyroid, especially insulin-producing tumors and islets of Langerhans where amyloid deposits are common.

Summary

Amyloid fibrils were studied from two different tissues of medullary carcinoma of the thyroid (MCT). The fibrils mainly consisted of a low molecular weight protein, AMmct, which was immunologically distinct and did not react with various antisera against known amyloid fibril proteins. A specific antiserum raised against the MCT amyloid proteins gave a reaction of identity with the degraded MCT amyloid fibrils from two patients, as well as with the isolated AMmct protein, but showed no reaction with other known amyloid proteins. The AMmct protein had a blocked N terminus, but the sequence analysis of a cyanogen bromide fragment revealed identity with human calcitonin in the 11 positions studied. Although the amino acid composition was similar, there were also distinct differences, and the mol wt of 5,700 daltons was considerably larger than that of calcitonin. For these reasons the AMmct protein may represent a prohormone of calcitonin.

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