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Role of the C-Terminal Fragment of Human Transthyretin in Abnormal Fibrillogenesis

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Abstract—Polypeptide chain fragments of recombinant transthyretin (TTR) with leucine-55 substituted by proline (L55P), which are involved in abnormal fibrillogenesis of this protein, were studied. No fibrils were produced in purified preparations of TTR(L55P) under the optimum conditions for fibrillogenesis but in absence of protease inhibitors. The ability of TTR for fibrillogenesis was lost because of a limited proteolysis resulting in detachment of the TTR polypeptide chain C-terminal fragment of ~18 amino acid residues in length. This proteolysis seemed to occur with involvement of a bacterial serine endopeptidase sohB (EC 3.4.21), which was identified in TTR preparations by the MALDI-TOF method. The presence of the C-terminal fragment of the TTR polypeptide chain seems to be crucial for production of abnormal fibrils.

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Key words: transthyretin, TTR(L55P), fibrillogenesis, aggregations, amyloidoses

Increasing attention is now being given to a type of severe human diseases, the amyloidoses. Amyloidoses are characterized by deposition in organs and tissues of insoluble protein aggregations, and this process is accompanied by progressing degenerative changes [1]. The depositions (amyloids) mainly consist of certain proteins producing fibrils, which, in turn, are responsible for sorption of other components [1]. Although amyloid deposits have been long known in medicine [2], comprehensive studies of abnormal protein fibrillogenesis became possible only recently. The ability for abnormal fibrillogenesis can manifest itself only under particular conditions, and it is associated with conformational changes in the protein [3]. These changes can be secondary (induction of abnormal conformers, as in the case of prion diseases) and primary, caused genetically or by disorders in protein folding [4].

The human transthyretin (TTR) protein is prone to fibrillogenesis [5]. TTR is thought to be involved in thyroxin transport in blood and cerebrospinal fluid [6] and in vitamin A transport mediated by the retinol-binding protein [5]. TTR *in vitro* can produce fibrils at low pH values [7] and high concentrations of salts in the solution [8]. Some mutant TTR forms are especially prone to fibrillogenesis [9]. Fibrils of mutant TTR forms have been found in such diseases as familial polyneuropathy [10], cardiomyopathy [9], and senile system amyloidoses [11]. TTR with leucine-55 substituted by proline (L55P) in the polypeptide chain is now considered to be the most dangerous amyloidogenic human protein [12].

TTR is a 55-kD homotetramer consisting of four identical subunits, each having a polypeptide chain of 127 amino acid residues [5]. The secondary structure of the TTR monomer is mainly represented by β-folds [13]. One molecule of tetrameric TTR can concurrently interact with two molecules of retinol-binding protein [5, 14], and the thyroxin-binding channel is located inside the center of the tetramer [5, 8, 15]. Several hypothetical models of TTR fibrillogenesis have been described [16-19]. Nevertheless, the TTR molecule is known to acquire the ability for fibrillogenesis only on disturbance of its quaternary structure. It is supposed that TTR dimers [16] or monomers [17-19] produced under certain conditions fail

Abbreviations: MALDI-TOF) Matrix-Assisted Laser Desorption/Ionization–Time of Flight; ThT) thioflavin T; TTR) transthyretin; TTR(L55P)) transthyretin with leucine-55 substituted by proline.

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to recover tetrameric structures but can interact to one another and form multimolecular aggregates. The aggregation starts by the "nucleation" stage, then prefibrils, or "protofibrils", are produced [20, 21] which grow to mature amyloid fibrils.

The literature data indicate the presence in the TTR polypeptide chain of two fragments, TTR(10-19) and TTR(105-115), which in themselves are capable of *in vitro* fibrillogenesis [22, 23]. According to X-ray crystallographic analysis, the secondary structure of every peptide inside the TTR molecule is a single β-strand, without bends or turns [22]. Electron microscopy shows that fibrils produced by the peptide TTR(10-19) are long (>1000 nm) spiral-like structures of ~10 nm in diameter [24]. Urea (3 M) destroys the ordered structure of these fibrils. However, such partially destroyed fibrils produce protofibrils of 3-4 nm in diameter, which are extremely resistant to denaturing agents [25]. Fibrils produced from the peptide TTR(105-115) are similar in morphology to the TTR(10-19) fibrils and are also resistant to denaturants [26].

The peptide TTR(10-19) is a component of the thyroxin-binding site [22] and the peptide TTR(105-115) is a part of the region responsible for monomer to monomer binding in the TTR tetramer structure. Although there is significant progress in studies on TTR fibrillogenesis, it is still unclear which regions of the polypeptide chain of TTR monomers are interacting to one another during the formation of amyloid fibrils.

MATERIALS AND METHODS

Isolation and purification of recombinant TTR(L55P). TTR(L55P) was isolated from the medium which had been used for culture of *Escherichia coli* BL21(DE3) cells transformed by the expressing construction TTR(L55P)/pET22b+ [27]. This vector was constructed to provide for synthesis of the protein together with the "leader peptide" responsible for its transfer from the cell into the LB medium, with subsequent detachment [27]. The cells were initially grown overnight in 10 ml of the ampicillin-containing (100 μg/ml) LB medium at 37°C. The resulting inoculate was diluted 100-fold in the fresh medium, and the culture was grown further at 37°C under aeration conditions until the $A_{600} = 1$. Then the TTR synthesis was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the final concentration of 1 mM, and the culture was grown overnight at 37°C under aeration. The bacterial cells were separated by centrifugation for 15 min at 10,000*g*, and the supernatant was subjected to ion-exchange chromatography on a column with DEAE A-50 Sephadex equilibrated with phosphate-buffered saline (PBS) containing 0.15 M NaCl and 25 mM sodium phosphate (pH 7.4). TTR(L55P) was eluted with a linear gradient of NaCl (150-350 mM). Fractions containing TTR(L55P) (the major peak) were concentrated on a PM-10 membrane. Then the material was gel filtered on a column (100 × 1.6 cm) with Toyopearl HW-50 equilibrated with PBS. The TTR-containing fractions were concentrated to 2 mg/ml and frozen at –70°C for long-term storage. The TTR concentration was determined spectrophotometrically using $\varepsilon_{280} = 16{,}315\ M^{-1}\cdot cm^{-1}$.

From 1 liter of the culture fluid, 20-30 mg of purified TTR was prepared. The TTR purification was monitored by PAGE [28]. Preparations with the TTR content >97% were chosen for experiments.

To study the electrophoretic mobility of TTR monomers, the samples were heated for 1 min on a water bath (at 100°C) in the presence of mercaptoethanol. The mobility of TTR tetramers was studied without heating the samples and treatment with mercaptoethanol.

***In vitro* production of TTR(L55P) fibrils.** To prepare fibrils, TTR(L55P) at the final concentration of 2 mg/ml was incubated with constant stirring in 15 mM sodium phosphate buffer (pH 6.0) supplemented with 0.2 M NaCl and 1 mM EDTA at 37°C for 12 h [29]. Samples were incubated with and without addition of a protease inhibitor ("Protease inhibitor cocktail" for the complete inhibition of proteases during extractions from animal and plant tissues or cells, yeast, and bacteria; Cat. No. 1,836,153, Boehringer Mannheim).

Fluorescence analysis of TTR fibrils on binding with thioflavin T (ThT). Fluorescence of fibrillogenesis was analyzed with a spectrofluorimeter in the Institute of Cytology (Russian Academy of Sciences, St. Petersburg) at the excitation and emission wavelengths of 435 and 482 nm, respectively [30]. The sample volume for the fluorescence determination was 400 μl. To 10 μl of the TTR fibril solution 390 μl of 25 mM sodium phosphate buffer (pH 8.0) and 1 μl of ThT aqueous solution (at the initial concentration of 40 μg/ml) were added.

Electron microscopy of TTR fibrils. Electron microscopy was performed with a JEM-100S electron microscope (JEOL, Japan) at the accelerating voltage of 80 kV. The samples were prepared by the standard method of negative contrasting: a drop of the sample under investigation was applied onto Parafilm, and from above a copper grid with a carbon substructure was placed onto the drop; after adsorption for 15 sec, the grid was washed twice in distilled water, contrasted with 1.5% sodium tungstophosphate solution (pH 7.4), and dried at room temperature.

MALDI-TOF mass spectroscopy of proteins and products of their trypsinolysis. Mass spectrometry was performed with a Bruker mass-spectrograph in the Institute of Physicochemical Medicine (Russian Federation Ministry of Health, Moscow).

Preparations of partially proteolyzed and unproteolyzed TTR were subjected to preparative PAGE under non-denaturing conditions. The control lane was cut off from the gel and stained with Coomassie R-250.

According to location of the stained band, the protein-containing lane was cut off from the unstained polyacrylamide gel. The protein was extracted from the gel during 24 h at 4°C with 0.1% trifluoroacetic acid, purified on ZipTipC18, and removed from the column with 75% acetonitrile.

For trypsinolysis in gel, the samples were subjected to SDS-PAGE with subsequent staining. The resulting peptide fingerprints of proteins were analyzed online using the MASCOT program at http://www.matrixscience.com.

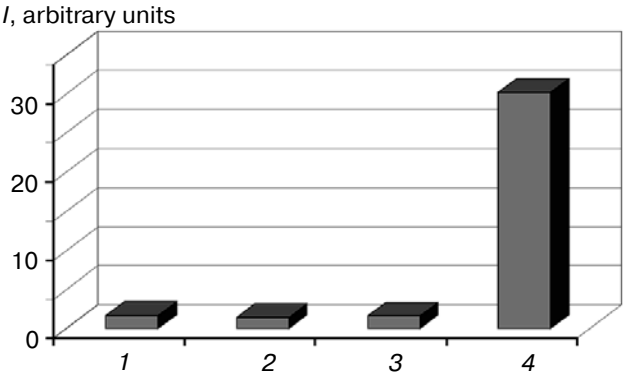

Fig. 1. Fluorescence of TTR and its fibrils on binding with ThT. Along the ordinate axis mean values of fluorescence obtained by three determinations are presented. *1*) Fluorescence of ThT; *2*) fluorescence of unincubated TTR on binding with ThT; *3*) fluorescence of TTR incubated in the absence of protease inhibitors on binding with ThT; *4*) fluorescence of TTR fibrils on binding with ThT.

RESULTS

Fibrils of recombinant TTR were prepared by long-term incubation at 37°C. Fluorescent analysis with ThT (Fig. 1) and electron microscopy (Fig. 2) revealed that typical fibrils appeared after 12 h of incubation (Fig. 2a). However, the fibrils arose and grew only if the inhibition medium was presupplemented with protease inhibitors. The fibrillogenesis was accompanied by opalescence, whereas the incubation of TTR samples without the inhibitors was associated with appearance of a flocculent precipitate. In electron microphotographs of such TTR samples only amorphous aggregations are detectable (Fig. 2b). Thus, suppression of fibrillogenesis in the samples incubated in the absence of protease inhibitors seems to be associated with destruction of TTR.

To elucidate the nature of fibrillogenesis suppression, the protein samples were subjected to electrophoresis before incubation under conditions of fibrillogenesis and after incubation in the presence and absence of protease inhibitors. The initial TTR in the presence of SDS migrated as a single band (Fig. 3, lane *2*), which corresponded to the protein tetramer. Heating the protein in the presence of SDS and mercaptoethanol promoted dissociation of TTR tetramers to monomers (Fig. 3, lane *3*). Electrophoresis of fibril preparations resulting after 72 h of incubation revealed a band that corresponded only to a 15-kD TTR monomer (Fig. 3, lanes *10* and *11*). No

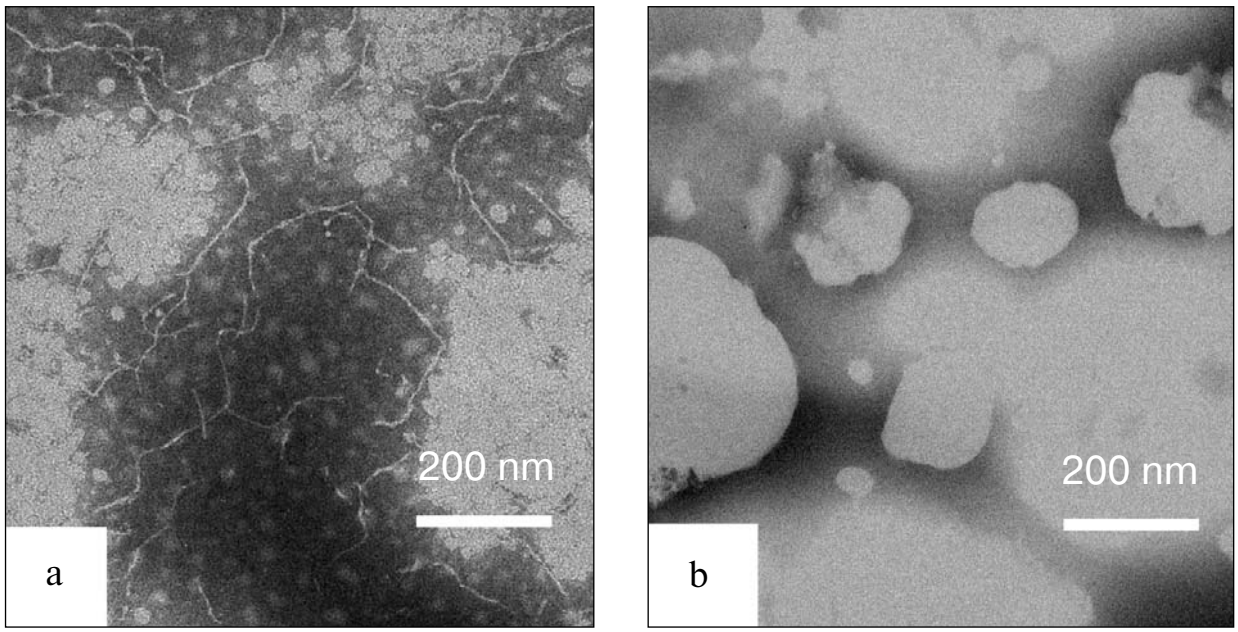

Fig. 2. Electron microphotographs of fibrils (a) and amorphous aggregations of TTR(L55P) (b). Negative contrasting with 1.5% sodium tungstophosphate solution.

tetramers were detected because the fibrils were dissolved only in the presence of mercaptoethanol. However, the electrophoretic mobility of the band corresponding to TTR tetramer was increased in the TTR preparations incubated in the absence of protease inhibitors (Fig. 3, lanes *6* and *8*), and a component could be detected with higher electrophoretic mobility than that of the initial TTR monomer (Fig. 3, lanes *5*, *7*, and *9*). The appearance of the new molecular forms was associated with a decrease in contents of the initial tetramers and monomers. After the incubation for 72 h, the initial protein was completely converted to forms with the lower molecular weights (Fig. 3, lanes *8* and *9*).

After incubation for 36 h, SDS-PAGE without preheating in the presence of mercaptoethanol revealed an increase in the mobility of the band corresponding to TTR tetramers (Fig. 3, lane *6*). Molecular weights of TTR tetramers were decreased after 72 h of incubation (Fig. 3, lane *8*).

After incubation for 24 h, SDS-PAGE of the preparations preheated in the presence of mercaptoethanol revealed an additional component with lower molecular weight (Fig. 3, lane *5*). After incubation for 36 h, the staining intensity of the band corresponding to the initial TTR monomers was decreased, but the staining intensity of the band corresponding to the component with the lower molecular weight was increased (Fig. 3, lane *7*). After 72 h of incubation, only TTR monomers with molecular weights lower than those of the initial monomers could be detected in the preparation (Fig. 3, lane *9*).

On incubation of TTR preparations under the same conditions but in the presence of protease inhibitors the above-described TTR degradation was not recorded (the pattern was similar to lanes *2* and *3* in Fig. 3).

MALDI-TOF mass spectroscopy. To determine true molecular weights of the components produced during incubation of TTR preparations without protease inhibitors and locate sites of the polypeptide chain cleavage, electrophoretically detectable individual TTR components were analyzed by mass spectroscopy.

The mass spectrum of the initial TTR preparation (Fig. 4) revealed three main peaks, which corresponded to proteins as follows: the 15.07-kD (major peak) (*3*) and the 14.36- and 12.86-kD minor components (*2* and *1*, respectively).

Trypsinolysis in gel: analysis of proteins by their peptide fingerprints. Samples of recombinant TTR, nonincubated (sample 1) and incubated for 72 h at 37°C without protease inhibitors (sample 2) were compared. The protein preparations were subjected to trypsinolysis in gel, and weights of the resulting fragments were determined by mass spectrometry. Using the MASCOT program and all databases available online, eight of 27 peptides in sample 1 present in the spectrum were ascribed to TTR (at 97% probability) (Table 1).

In sample 2, 18 peptides were found, and seven of them belonged to TTR (at 97% probability) (Table 2).

The amino acid composition of the peptides in samples 1 and 2 was compared with the amino acid sequence of TTR from the pbd database (www.ncbi.nlm.nih.gov) (Tables 1 and 2), and TTR from the sample 1 was found

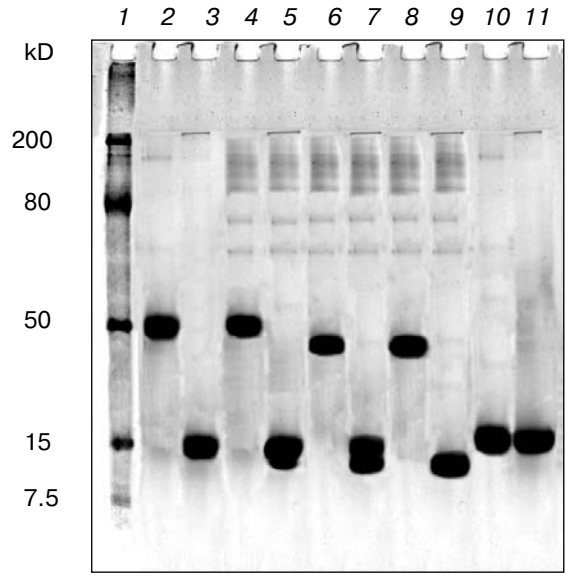

Fig. 3. Electrophoresis under denaturing conditions: *1*) molecular weight markers; *2*, *4*, *6*, *8*) TTR tetramers; *3*, *5*, *7*, *9*, *10*, *11*) TTR monomers.

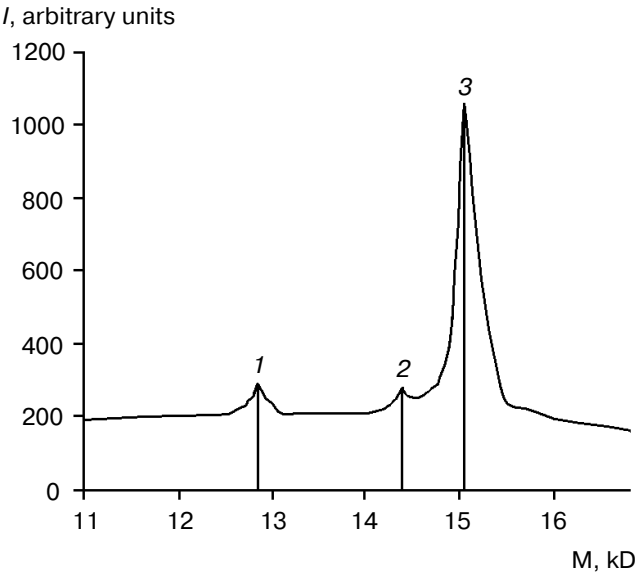

Fig. 4. Mass spectrum of recombinant TTR(L55P) preparation containing both the full-size protein (peak *3*) and partially proteolyzed forms of TTR (peaks *1* and *2*).

Table 1. Peptide fingerprint of TTR in the nonincubated preparation

Start–end	Expected value of *M*, daltons	Calculated value of *M*, daltons	Δ, daltons	Peptide structure
10-21	1342.61	1342.75	–0.14	**K.CPLMVKVLDAVR.G**
22-34	1365.70	1365.75	–0.05	**R.GSPAINVAVHVFR.K**
35-48	1521.66	1521.71	–0.05	**R.KAADDTWEPFASGK.T**
36-48	1393.57	1393.61	–0.04	**K.AADDTWEPFASGK.T**
49-70	2438.14	2438.11	0.03	**K.TSESGEPHGLTTEEEFVEGIYK.V**
49-76	3123.46	3123.48	–0.02	**K.TSESGEPHGLTTEEEFVEGIYKVEIDTK.S**
81-103	2450.20	2450.20	0.00	**K.ALGISPFHEHAEVVFTANDSGPR.R**
105-127	2488.26	2488.27	–0.01	**R.YTIAALLSPYSYSTTAVVTNPKE.–**

Notes: The peptides identified in the TTR preparation are shown in bold print and underlined. The primary structure of TTR: GPTGTGESK-**CPLMVKVLDAVRGSPAINVAVHVFRKAADDTWEPFASGKTSESGEPHGLTTEEEFVEGIYKVEIDTK**SYWK**ALGISPFHEHAEVVF-TANDSGPRRYTIAALLSPYSYSTTAVVTNPKE**. Molecular weights of unidentified peptides found in the sample: 409.07, 410.05, 428.06, 439.06, 468.00, 469.04, 497.99, 501.01, 525.52, 540.99, 570.92, 583.27, 665.02, 693.97, 723.92, 995.53, 1262.57, 2043.92, 2059.93.

Table 2. Peptide fingerprint of TTR in the preparation incubated without protease inhibitors

Start–end	Expected value of *M*, daltons	Calculated value of *M*, daltons	Δ, daltons	Peptide structure
10-21	1342.61	1342.75	–0.14	**K.CPLMVKVLDAVR.G**
16-21	671.39	671.40	–0.01	**K.VLDAVR.G**
22-34	1365.71	1365.75	–0.04	**R.GSPAINVAVHVFR.K**
35-48	1521.67	1521.71	–0.04	**R.KAADDTWEPFASGK.T**
36-48	1393.58	1393.61	–0.03	**K.AADDTWEPFASGK.T**
49-76	3123.43	3123.48	–0.05	**K.TSESGEPHGLTTEEEFVEGIYKVEIDTK.S**
81-103	2450.15	2450.20	–0.05	**K.ALGISPFHEHAEVVFTANDSGPR.R**

Notes: The peptides identified in the TTR preparation are shown in bold print and underlined. The primary structure of TTR: GPTGTGESK-**CPLMVKVLDAVRGSPAINVAVHVFRKAADDTWEPFASGKTSESGEPHGLTTEEEFVEGIYKVEIDTK**SYWK**ALGISPFHEHAEVVF-TANDSGPRR**YTIAALLSPYSYSTTAVVTNPKE. Molecular weights of unidentified peptides found in the sample: 379.15, 380.17, 401.12, 441.04, 443.04, 568.13, 583.29, 650.05, 856.52, 1234.64, 1493.71, 2043.94.

to be one residue longer than TTR from sample 2. The peptide obtained as a result of full trypsinolysis of TTR found in sample 1 and absent in sample 2 was situated on the C-end of the TTR polypeptide chain and consisted of 23 amino acid residues (Table 1).

Because TTR used in our experiments was synthesized in a bacterial system, minor amounts of bacterial proteins could be present in the samples. We analyzed the peptide composition of samples 1 and 2 to identify proteins from *E. coli*. The MASCOT program was supplemented with the function of detecting *E. coli* proteins only containing the same peptides as found in samples 1 and 2. Some of the peptides in these samples were shown (with 25% probability) (Table 3) to be constituents of the bacterial protease sohB (EC 3.4.21) of serine endopeptidases. The protease sohB was identified by five peptides (Table 3), and only one of them corresponded in molecular weight to the peptide found among the products of TTR trypsinolysis.

DISCUSSION

Recombinant TTR with the L55P substitution studied by us is the most prone to fibrillogenesis among known human proteins. This mutant TTR can produce fibrils even during the elimination stage if special precautions are not taken. However, if TTR preparations were incubated under conditions favorable for fibrillogenesis but in the absence of protease inhibitors, no fibrils were

Table 3. Peptide fingerprint of the protease sohB

Start–end	Expected value of *M*, daltons	Calculated value of *M*, daltons	Δ, daltons	Peptide structure
14-36	2359.33	2359.47	–0.14	**K.IVTVVLAIAAIAAIIVNVAQRNK.R**
40-52	1521.67	1520.75	0.92	**R.GELQVNNLSEQYK.E**
92-109	2058.98	2059.11	–0.13	**K.LGEVATDSKPRVWVLDFK.G**
260-264	664.00	663.37	0.63	**K.DFVKR.M**
320-324	671.39	671.41	–0.02	**K.RLIDR.F**

Notes: The peptides identified in the preparation are shown in bold print and underlined. The primary structure of sohB (EC 3.4.21): MELLSEYGLFLAK**IVTVVLAIAAIAAIIVNVAQRNK**RQR**GELQVNNLSEQYK**EMKEELAAALMDTHQQKQWHKAQKKKHKQEAKAAKAKAK**LGEVATDSKPRVWVLDFK**GSMDAHEVNSLREEITAVLAAFKPQDQVVLRLESPGGMVHGYGLAASQLQRLRDKNIPLTVVDKVAASGGYMMACVADKIVSAPFAIVGSIGVVAQMPNFNRFLKSKDIDIELHTAGQYKRTLTLLGENTEEGREKFREELNETHQLFK**DFVKR**MRPSLDIEQVATGEHWYGQQAVEKGLVDEINTSDEVILSLMEGREVVNVRYMQRK**RLIDR**FTGSAAESADRLLLRWWQRGQKPLM. Molecular weights of unidentified peptides found in the sample: 409.07, 410.07, 439.06, 468.01, 469.03, 498.00, 525.51, 540.97, 548.96, 550.96, 570.93, 583.29, 646.99, 650.00, 675.97, 693.96, 995.50, 1366.70, 1394.58, 2043.99, 2439.20, 2451.26.

produced. Moreover, during the incubation protein derivatives with lower molecular weights appeared, which was a direct indication of limited proteolysis. Monomer constituents of the tetramer form were degraded by proteolysis, and no dissociation products were found. And data of electrophoresis suggested a step-wise degradation of monomers, with successive detachment of two small polypeptide fragments.

It seems that the limited proteolysis most likely initiated due to admixture of bacterial protease prevents nucleation and growth of fibrils. The major part of the protein was aggregated disorderly. During the incubation period, a fragment with molecular weight lower than that of the TTR subunit was detached from the TTR tetramer, and this suggested the lack of destruction of the quaternary structure of the protein but the proteolytic detachment of terminal fragment from every TTR monomer.

Because the elimination of small peptide fragments inhibited fibrillogenesis, it was interesting to elucidate what region of the monomers was destroyed and what peptides were separated as a result of the limited proteolysis. This was studied by mass spectrometry of TTR monomers and products of their trypsinolysis. According to analysis of the initial recombinant TTR, the molecular weight of the monomer corresponded to the primary structure described in the literature. In addition to the major 15.07-kD component, 14.36- and 12.86-kD degradation products were detected by electrophoresis in the samples incubated without protease inhibitors. Thus, mass spectrometry allowed us to detect initial stages of proteolysis much earlier than electrophoresis. Unfortunately, we failed to record satisfactory mass spectra of the monomers isolated electrophoretically from the samples incubated under conditions favorable for fibrillogenesis without protease inhibitors. Therefore, to find sites of the proteolytic attack, we used trypsinolysis of monomers followed by mass spectrometry of the resulting peptides. In addition to elucidation of the limited proteolysis type, this approach allowed us to identify the protein subjected to fragmentation by comparison with the peptide sets of the known proteins from databases.

Analysis of peptides resulting from trypsinolysis of the full-size and partially proteolyzed TTR preparations revealed that the peptides corresponding to the partially proteolyzed TTR lacked the C-terminal fragment YTIAALLSPYSYSTTAVVTNPKE. Thus, it was suggested that a peptide consisting of no more than 23 amino acids should be detached from the C-end of the TTR polypeptide chain. Mass spectroscopy allowed us to locate sites affected by protease and to determine the number of amino acids detached during proteolysis. The spectrum of molecular weights of proteins in the sample with the started limited proteolysis of TTR suggested to us that a 710-dalton fragment was the first one detached from the amino acid sequence of TTR, and this fragment corresponded to the C-terminal peptide consisting of six (VTNPKE) or seven (VVTNPKE) amino acid residues. Then a 1500-dalton fragment corresponding to 11 (LSPYSYSTTA(V)) or 12 (LLSPYSYSTTA(V)) amino acid residues seemed to be detached. Consequently, the full length of the detached fragment (considering the measurement error) was 17-18 amino acid residues, and the mass spectrometry data were in good agreement with the above-described data of SDS-PAGE.

Thus, the presence of the C-terminal fragment of ~18 amino acid residues in length is a necessary prerequisite for formation of TTR(L55P) fibrils. It seems that attacking of just this C-terminal region of the TTR molecule will result in creation of harmless inhibitors of abnormal TTR fibrillogenesis and development of new

approaches based on molecular biology for treatment of such diseases as amyloidoses.

In addition to determination of the detachable peptide fragments of TTR, mass spectrometry of trypsinolysis products allowed us to rather reliably identify a protease involved in the limited degradation of TTR. Among trypsinolysis products minor peptides were found (Table 1), which according to computer-aided analysis were likely to be derivatives of the previously described *E. coli* protease [31]. Unfortunately, this protease has not been isolated and its specificity remains to be established. It is only known to be a serine protease.

REFERENCES

1. Koo, E. H., Lansbury, P. T., Jr., and Kelly, J. W. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 9989-9990.
2. Dwulet, F. E., and Benson, M. D. (1983) *Biochem. Biophys. Res. Commun.*, **114**, 657-662.
3. Lai, Z., Colon, W., and Kelly, J. W. (1996) *Biochemistry*, **35**, 6470-6482.
4. Dobson, C. M. (1999) *Trends Biochem. Sci.*, **24**, 329-332.
5. Kelly, J. W., and Lunsbury, P. T. (1994) *Amyloid*, **1**, 186-205.
6. Zheng, W., Lu, Y., Lu, G., Zhao, Q., Cheung, O., and Blaner, W. (2001) *Toxicol. Sci.*, **61**, 107-114.
7. Skoulakis, S., and Goodfellow, J. M. (2003) *Biophys. J.*, **84**, 2795-2804.
8. Hornberg, A., Hultdin, U. W., Olofsson, A., and Sauer-Eriksson, A. E. (2005) *Biochemistry*, **44**, 9290-9299.
9. Joao, M., and Saraiva, M. (1995) *Hum. Mutat.*, **5**, 191-196.
10. Bergquist, J., Andersen, O., and Westman, A. (2000) *Clin. Chem.*, **46**, 1293-1300.
11. Misu, K., Hattori, N., Nagamatsu, M., Ikeda, S., Ando, Y., Nakazato, M., Takei, Y., Hanyu, N., Usui, Y., Tanaka, F., Harada, T., Inukai, A., Hashizume, Y., and Sobue, G. (1999) *Brain*, **122**, 1951-1962.
12. Lashuel, H. A., Wurth, C., Woo, L., and Kelly, J. W. (1999) *Biochemistry*, **38**, 13560-13573.
13. Serag, A. A., Altenbach, C., Gingery, M., Hubbel, W. L., and Yeatis, T. O. (2002) *Natl. Struct. Biol.*, **9**, 734-739.
14. White, J. T., and Kelly, J. W. (2001) *Proc. Natl. Acad. Sci. USA*, **98**, 13019-13024.
15. Rosen, H. N., Moses, A. C., Murell, J. R., Liepnieks, J. J., and Benson, M. D. (1993) *J. Clin. Endocrinol. Metab.*, **77**, 370-374.
16. Olofsson, A., Ippel, H. J., Baranov, V., Horstedt, P., Wijmenga, S., and Lundgren, E. (2001) *J. Biol. Chem.*, **276**, 39592-39599.
17. Quintas, A., Saraiva, M. J., and Brito, R. M. (1999) *J. Biol. Chem.*, **274**, 32943-32949.
18. Quintas, A., Vaz, D. C., Cardoso, I., Saraiva, M. J., and Brito, R. M. (2001) *J. Biol. Chem.*, **276**, 27207-27213.
19. Hurshman, A. R., White, J. T., Powers, E. T., and Kelly, J. W. (2004) *Biochemistry*, **43**, 7365-7381.
20. Lindgren, M., Sorgjerd, K., and Hammarstrom, P. (2005) *Biophys. J.*, **88**, 4200-4212.
21. Serpell, L. C., Sunde, M., Benson, M. D., Tennent, G. A., Pepys, M. B., and Fraser, P. E. (2000) *J. Mol. Biol.*, **300**, 1033-1039.
22. Jarvis, J. A., Craik, D. J., and Wilce, M. C. (1993) *Biochem. Biophys. Res. Commun.*, **192**, 991-998.
23. Jaroniec, C. P., MacPhee, C. E., Astrof, N. S., Dobson, C. M., and Griffin, R. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 16748-16753.
24. Jaroniec, C. P., MacPhee, C. E., Bajaj, V. S., McMahon, M. T., Dobson, C. M., and Griffin, R. (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 711-716.
25. MacPhee, C., and Dobson, C. M. (2000) *Chem. J. Mol. Biol.*, **297**, 1203-1215.
26. Dirix, C., Meersman, F., MacPhee, C., Dobson, C. M., and Heremans, K. (2005) *J. Mol. Biol.*, **347**, 903-909.
27. Schwarzman, A. L., Tsiper, M., Wente, H., Wang, A., Vitek, M. P., Vasiliev, V., and Goldgaber, D. (2004) *Amyloid*, **11**, 1-9.
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular Cloning*, USA Cold Spring Harbor Laboratory Press, N. Y., pp. 174-175.
29. Hammarstrom, P., Jiang, X., Hurshman, A. R., Powers, E. T., and Kelly, J. W. (2002) *Proc. Natl. Acad. Sci. USA*, **99**, 16427-16432.
30. LeVine, H. (1993) *Protein Sci.*, **2**, 404-410.
31. Baird, L., Lipinska, B., Raina, S., and Georgopoulos, C. (1991) *J. Bacteriol.*, **173**, 5763-5770.