BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **249,** 26–30 (1998)
ARTICLE NO. RC989097

Post-translational Modification of Transthyretin in Plasma

Hisayasu Terazaki,* Yukio Ando,*,† Ole Suhr,† Per-Ingvar Ohlsson,‡ Konen Obayashi,*
Taro Yamashita,§ Shin-ichi Yoshimatsu,* Moritaka Suga,*
Makoto Uchino,§ and Masayuki Ando*,[1,2]

**First Department of Internal Medicine and §Department of Neurology, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860, Japan; †Department of Medicine, University of Umeå, S-901 85 Umeå, Sweden; and ‡Department of Medical Biochemistry and Biophysics, University of Umeå, S-901 87 Umeå, Sweden*

Received June 26, 1998

To determine the behavior of transthyretin (TTR) in blood circulation, TTR purified from normal subjects' plasma was injected to rats, and blood and urine were collected time dependently. Although TTR in plasma was proven to be a predominantly cysteine (Cys) conjugated form by electrospray ionization mass spectrometry (ESI-MS) analysis, it was gradually converted into free, 32 Da (dihydroxylation), 80 Da (phosphorylation), and 306 Da (glutathionylation), increased forms in molecular weight of TTR. The plasma levels of TTR were decreased in a time-dependent manner with the half life of 72.4 min. No secretion of TTR into the urine was observed by ESI-MS. In conclusion, this method can be simply performed without loading a radioactive molecule to the targeted protein. It offers a possibility to determine natural protein behaviors in the blood stream.

Among the hereditary systemic amyloidosis, familial amyloidotic polyneuropathy (FAP) Met30, is the most common (1, 2). The disease is characterized by a progressive painful peripheral neuropathy, though symptoms from the gastrointestinal tract, heart, kidney, eye, and the autonomic nervous system are also present. FAP Met30 is concentrated to restricted endemic areas, such as the northern part of Sweden, the Arao and Nagano districts of Japan, and the northern part of Portugal (1-4). Although FAP Met30 is the most common type of FAP, more than 60 reports of other single or double amino acid substitutions of transthyretin (TTR) exist (5), of which the majority is associated with various clinical manifestations of amyloidosis (6-8). Some of the non-TTR Met 30 related amyloidosis is sporadic and not restricted to small endemic areas (6, 7), and has therefore been difficult to diagnose and classify. Moreover, the clinical symptoms may differ from the most common forms of TTR related amyloidosis, FAP Met30 (8, 9). Thus, metabolism or behavior of TTR may differ in each mutant protein in the body.

Two techniques for analyzing variant forms of proteins are available, matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI/TOFS) and electrospray ionization mass spectrometry (ESI-MS). The latter technique is often combined with high performance liquid chromatography (HPLC). It has previously been reported that abnormal alpha and gamma chain and variant forms of proteins were detected by MALDI/TOFS (10, 11) and HPLC/ESI-MS (12), respectively. Recently, we have developed a rapid and reliable screening method for identifying genetic variants of TTR in FAP by using a centrifugal concentrator device and ESI-MS (13). With this method, several components of TTR, such as unmodified, phosphorylation, cysteinylation, glutathionylation forms of wild type and Met30 TTR were observed in healthy control subjects' and patients' sera (13, 14). We can determine the changes in protein modifications in cerebro-spinal fluid as well as plasma with that method (13, 14).

The purpose of the present study is to determine the behavior of purified TTR in vivo. By ESI-MS, the changes in the modifications and a half life of TTR were examined in plasma and urine after injecting purified human TTR to rats.

[1] To whom correspondence should be addressed at First Department of Internal Medicine, Kumamoto University School of Medicine, 1-1-1 Honjo, Kumamoto 860-0811, Japan. Fax: +81 96 371 0582. E-mail: yukio@kaiju.medic.kumamoto-u.ac.jp.

[2] Dr. Yukio Ando worked temporarily as a visiting professor at Department of Medicine, Umeå University Hospital.

Abbreviations used: FAP, familial amyloidotic polyneuropathy; TTR, transthyretin, Cys, cysteine; MALDI/TOFS, matrix-assisted laser desorption ionization/time-of-flight mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; HPLC, high performance liquid chromatography; Fr. IV, fraction IV.

0006-291X/98 $25.00

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 250-300 g used for all the in vivo experiments were obtained from Japan SLC (Shizuoka, Japan). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques.

Purification of normal TTR from sera. Fraction IV(Fr. IV) was purified from 200 l of normal human plasma by Cohn's ethanol fraction method. The source plasma met the standards then in effect for source plasma (negative for hepatitis B surface antigen, anti-hepatitis C antibody, and anti-HIV-I-antibody, and alanine aminotransferase level less than twice of the upper limit of normal). Ten kg of Fr. IV was suspended in 30 l of 0.15 M NaCl, 0.02 M Tris-HCl buffer, pH 7.6, and the mixture was stirred for 1 h at 4°C. After filtration using a filter (TOYO-roshi No. 2, Japan) with celite, the pH of the collected filtrate was adjusted again to pH 7.6, at 4°C, and the solution was kept at 60°C for 10 h to inactivate viruses. After the incubation, the pH of the solution was readjusted to pH 7.6, 150 g/l of polyethyleneglycol 4000 (Pharmacia, Uppsala, Sweden) was added and stirred for 1 h at 4°C. Then, the mixture was centrifuged at 4000 rpm for 30 min to separate the precipitate. The pH of the supernatant was adjusted to 7.6. One kg of DEAE-Sepharose fast flow (Pharmacia, Uppsala, Sweden) was added to this solution and mixed. The resin was then washed with 3 resin volumes of 0.02 M Tris, 0.1 M NaCl, pH 7.6, on a glass filter. After washing, 1 l of 0.02 M Tris, 0.5 M NaCl, Tris-HCl buffer, pH 7.6, was used to elute the TTR. After the procedure, 175 g/l of NaCl was added to the TTR elution and the pH was readjusted to 7.6. 1.5 kg of Phenyl-Sepharose High Performance (Pharmacia, Uppsala, Sweden) was added to the TTR containing mixture. The resin was washed with three resin volumes of 0.02 M Trs, 3 M NaCl, pH 7.6 on a glass filter. The TTR containing fractions were collected as the pass through and the resulting solution was concentrated and dialyzed by ultrafiltration (Model Minisette, 10 kD-OMEGA, Filtron, USA).

In vivo experiment. *In vivo* experiments were performed between 8:00 AM and 12:00 noon after the rats fasted for 12 h. Under pentobarbital anesthesia (50 mg/kg body weight), heparinized animals (1000 u/kg body weight) were intravenously administered with 3 mg / 200 μl saline of TTR into left femoral vein. At varying times after administration, 0.1 ml of blood samples was collected from the left femoral vein. After 3 h of administration, animals were exsanguinated by bleeding from the lower part of the abdominal artery.

TTR isolation from the rats' sera. Fifty milliliters test serum was mixed with 20 ml of anti-TTR antibody (Dako, Dakopatts, Glostrup, Denmark). The generated precipitate was centrifuged at 9000*g* for 5 min and washed with 100 ml water three times at 4°C. The precipitate was dissolved in 50 ml of 4% acetic acid and 4% acetonitrile in water and the solution was passed through a 1000 kDa centrifugal concentrator (Pall Filtron Co., Northborough, MA, USA) to obtain the dissociated TTR from the antibody in the pass through fraction. The centrifugal devices were washed 3 × 100 ml with the same solution.

Plasma half life of TTR. Amount of injected TTR in plasma of rats in each time spot was determined by measuring the intensity of ESI-MS data. A plasma half life was determined by semilogarythmic plotting of each data.

Mass spectrometry. The ion spray mass analysis were performed in a API I single quadrupole mass spectrometer (Sciex, Perkin-Elmer Corp., Thornhill, Ontario, Canada). The samples were infused at 5 ml min^{-1} with a Harvard Apparatus syringe pump (Harvard Apparatus, Southnatick, Massachusetts, USA) into the ion spray interface. The spray chamber was maintained at 54°C during the analysis, and orifice voltage between 70 and 80V. The spectra were acquired in the positive mode with a step size of 0.5 mm and a dwell time of 1.0 msec per step. Molecular masses of the HPLC isolated peptides were determined with MacSpec 3.3 (Sciex software). In most cases the saved spectra were obtained by summing 10 spectra in the multichannel analyzer mode.

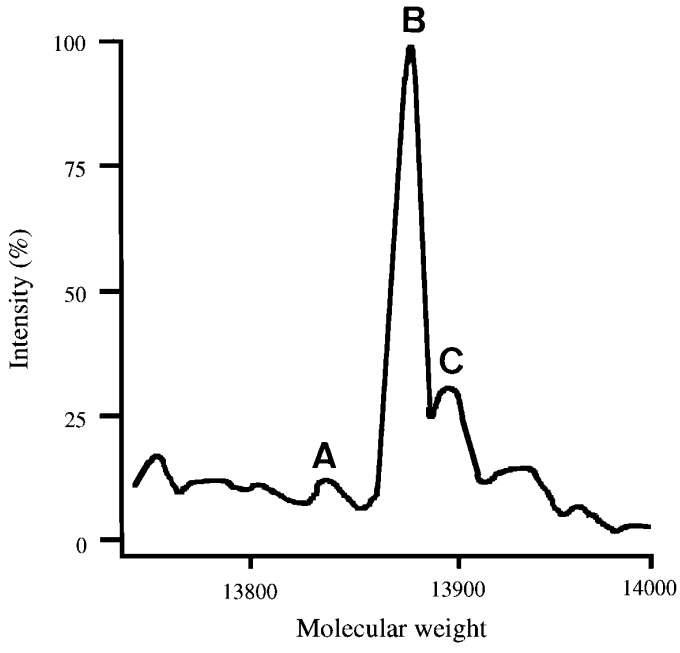

FIG. 1. Analysis of purified protein by ESI-MS. The purified protein was charged to ESI-MS as described in the text. (A) Free form, (B) Cys-conjugated (cysteinylation), and (C) sodium conjugated forms of TTR (13).

RESULTS

Preparation of purified normal TTR. Wild type TTR was prepared from bacterial- and viral- free normal subjects' sera as described above. SDS gel electrophoresis revealed that in the presence of mercaptoethanol, monomer of TTR were mainly recognized and no other special proteins were contaminated (data not shown). The purity of TTR was calculated to be more than 95% by high performance liquid chromatography (HPLC) analysis. By the analysis of ESI-MS, the peak of 13881 Da (Cysteinylation of TTR) was predominantly detected (Fig. 1).

Detection of TTR in rats' sera. The purified human TTR injected from the left femoral vein of rats was detected in plasma. The several peaks, such as 13715, 13792, 13840, 13938, and 14067 Da in molecular weights were detected after injection of TTR (Fig. 2). The peak of 13881 Da in molecular weight of the purified protein was gradually decreased after the injection and peaks of 13761, 13840, and 14067 Da were increased as time-dependent manner (Fig. 3). No obvious peaks were detected in urinary samples (data not shown).

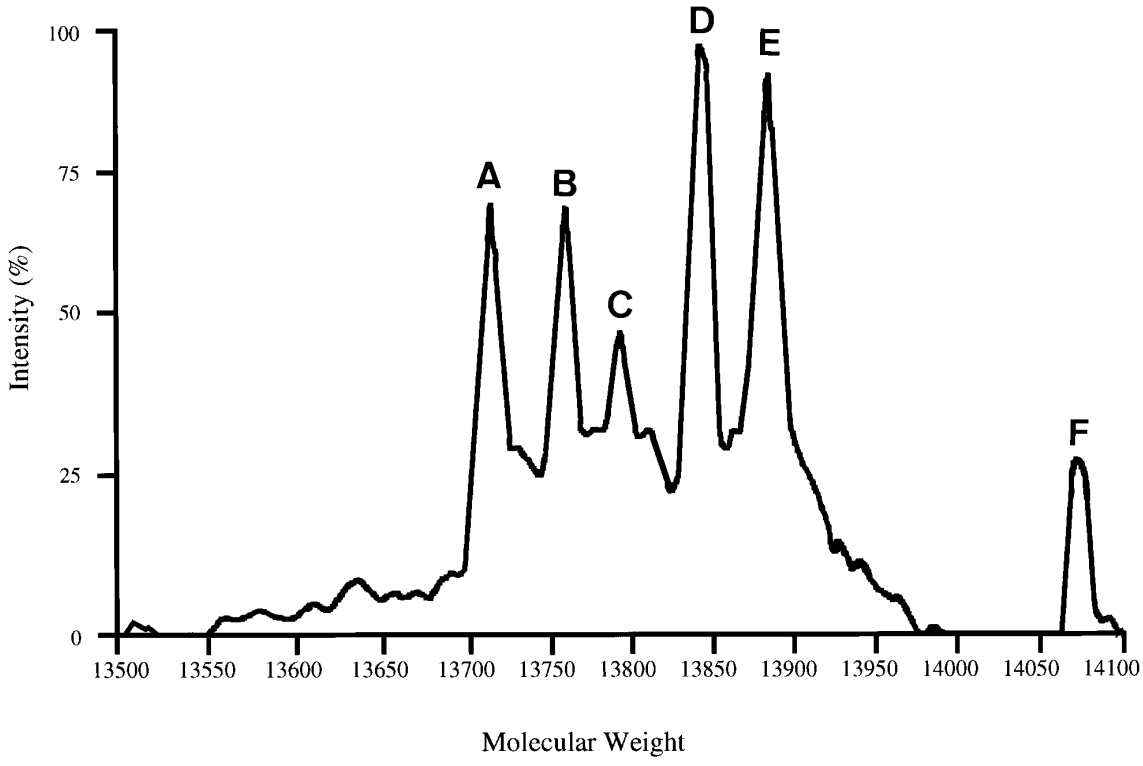

FIG. 2. A typical serum pattern at 180 min after TTR injection. (A) Unknown peak, (B) free form, (C) dehydration, (D) phosphorylation, (E) cysteinylation, and (F) glutathionylation of TTR.

Fate of intravenously administered TTR in rats. To determine the plasma half life of TTR, blood was collected as described above. TTR administered to rats was decreased from the blood circulation time-dependent manner and by the semilogarythmic plotting the data revealed 72.4 min of the plasma half life (Fig. 4).

DISCUSSION

We have presented in this report that TTR modifications and the plasma half life could be determined in rats' sera by ESI-MS. We previously reported the plasma half life and organ distributions of human TTR by injecting ^{125}I-labeled purified human TTR to rats (15). Compared with those data, the plasma half life was significantly shorter and no TTR could not be detected in the urine in this examination. It is well documented that TTR forms tetramer and undergoes no

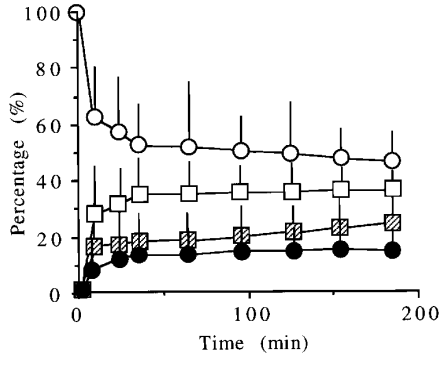

FIG. 3. Change in TTR modifications in rats. ○, cysteinylation; ●, glutathionylation; □, phosphorylation; and ▨, free forms of TTR. The data were expressed as the average SD of three animals.

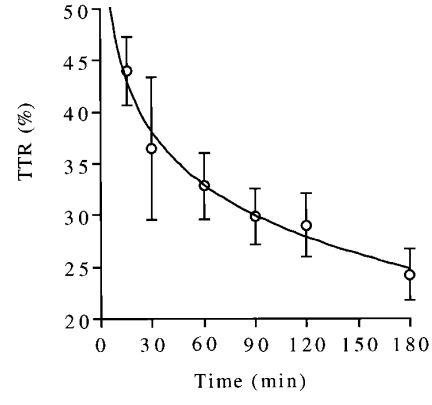

FIG. 4. Fate of injected TTR in blood. Three milligrams of purified human TTR was injected from the left femoral vein of rats. The data were expressed as the average SD of three animals. From the semilogarythmic plots of the data, a value of 72.4 min was calculated for the serum half life of TTR in the circulation of rats.

glomerular filtration in normal state (16). Since significant portion of ^{125}I can be dissociated form ^{125}I-TTR in blood stream, there is a possibility that free form of ^{125}I with no TTR could be counted as ^{125}I-TTR in the urine in the previous paper (15, 17).

The method employed in this experiment can detect the real protein behavior. We can check the native protein behavior without loading an additional molecular weight of a radioisotope and epitope to conjugate a radioactive molecule to a protein, so possible change in an artificially modified behavior and unnatural metabolism of a protein can be eliminated. Since the rat TTR shows about 80 % homology of the protein structure, human TTR injected to rats mimics human TTR behavior in human body (18).

It should be noted that, until now, more than 60 points of single or double amino acid substitutions of TTR exist (5, 19) and clinical symptoms vary depending on the specific forms of TTR mutations, although polyneuropathy is the dominating feature (8, 9). This indicates that the affinity of mutated TTRs for the amyloid targeted organs varies for only the difference of one point TTR mutation. Thus, a loading of extra molecules on the examining protein may be critical for determining the protein behavior.

By ESI-MS, various TTR modifications could be determined: According to the previous reports, 13761, 13792, 13840, 13938, and 14067 Da in molecular weights correspond to free form, dihydroxylation, phosphorylation, Cysteine-Gly conjugation, and glutathionylation forms, respectively (20, 21). The increase in glutathionylation after injecting the protein may be explainable because the activity of-GTP is less in rats than that of human, resulting in the increase in this type of modification (22). However, the reason why free and phosphorylation forms of TTR was increased after injection of TTR is not known. Especially, the increase in the peak of phosphorylation was more obvious in turpentine injected rats (data not shown). This information suggests that an increase in this peak may be correlated with inflammation in vivo. Extracellular phosphorylation has been focused in the recent research (23). By unknown mechanism(s), TTR may undergo phosphorylation in the circulation of rats. Further examination should be needed to clarify this concept.

Since the employed antibody showed a little affinity for intrinsic rat's TTR (24), the peaks for reflecting on the rat's TTR could not be detected by this method. We may be able to determine the rat's intrinsic proteins' behavior if suitable antibodies were available or could be produced.

In conclusion, this method can be performed without labeling a targeted protein with a radioactive molecule and epitope to conjugate a radioactive molecule to a protein. The method is simple and offers a possibility to determine natural protein behaviors in the blood stream. Other proteins should be possible to detect in serum by the presented method if suitable antibodies were obtained.

ACKNOWLEDGMENTS

This study was supported by grants from the patients association (FAMY), the Medical Faculty, Umeå University, Västerbottens Health District, and The Swedish Council for Planning and Coordination of Research (FRN).

REFERENCES

1. Araki, S. (1986) *in* Amyloidosis (Rijswijk, M. H., Ed.), pp. 195–218, Nijhoff, Dordrecht, The Netherlands.
2. Ando, Y., Araki, S., and Ando, M. (1993) *Intern. Med.* **32,** 920–922.
3. Drugge, U., Anderson, R., Chizari, F., Danielsson, M., Holmgren, G., Sandgren, O., and Sousa, A. (1993) *J. Med. Genet.* **30,** 382–392.
4. Sequeiros, J., Sousa, A., and Coelho, I. (1990) *in* Amyloid and Amyloidosis (Natvig, J. B., Forre, O., Husby, G., Husebekk, A., Skogen, B., Slettern, K., and Westermark, P., Eds.), pp. 687–690, Academic, Dordrecht.
5. Petersen, R. B., Goren, H., Cohen, M., Richardson, S. L., Tresser, N., and Lynn, A. (1997) *Ann. Neurol.* **41,** 307–313.
6. Murakami, T., Atsumi, T., Maeda, S., Tanase, S., Ishikawa, K., Mita, S., Kumamoto, T., Araki, S., and Ando, M. (1992) *Biochem. Biophys. Res. Commun.* **187,** 397–403.
7. Saeki, Y., Ueno, S., Takahashi, N., Soga, F., and Yanagihara, T. (1992) *FEBS Lett.* **10,** 35–37.
8. Ueno, S., Uemichi, T., Takahashi, N., Yorifuji, S., and Tarui, S. (1990) *Biochem. Biophys. Res. Commun.* **169,** 1117–1121.
9. Saeki, Y., Ueno, S., Yorifuji, S., Sugiyama, Y., and Matsuzawa, Y. (1991) *Biochem. Biophys. Res. Commun.* **180,** 380–385.
10. Terukina, S., Matsuda, M., Hirata, H., Takeda, Y., Takao, T., and Shimonishi, Y. (1988) *J. Biol. Chem.* **263,** 13579–13587.
11. Nakanishi, T., Shimizu, A., Oamoto, N., Ingendoh, A., and Kanai, M. (1995) *J. Am. Soc. Mass. Spectrom.* **6,** 854–859.
12. Kishikawa, M., Nakanishi, T., Miyazaki, T., Shimizu, A., Nakazato, M., Kangawa, K., and Matsuo, H. (1996) *J. Mass. Spectrom.* **31,** 112–114.
13. Ando, Y., Ohlsson, P. I., Suhr, O., Nyhlin, N., Yamashita, T., Holmgren, G., Danielsson, Å., Sandgren, O., Uchino, M., and Ando, M. (1996) *Biochem. Biophys. Res. Commun.* **228,** 480–483.
14. Ando, Y., Suhr, O., Ohlsson, P. I., Yamashita, T., Holmgren, G., Obayashi, K., Terazaki, H., Mambule, C., Uchino, M., and Ando, M. (1997) *Neurosci. Lett.* **238,** 123–126.
15. Ando, Y., Ikegawa, S., Miyazaki, A., Inoue, M., Morino, Y., and Araki, S. (1990) *Arch. Biochem. Biophys.* **274,** 87–93.
16. Benson, M., and Uemichi, T. (1996) *Amyloid Int. J. Exp. Clin. Invest.* **3,** 44–56.
17. Makover, A., Moriwaki, H., Ramakrishnan, R., Saraiva, M. J., Blaner, W. S., and Goodman, D. S. (1988) *J. Biol. Chem.* **263,** 8598–8603.
18. Sundelin, J., Melhus, H., Das, S., Eriksson, U., Lind, P., Tragardh, L., Peterson, P. A., and Rask, L. (1985) *J. Biol. Chem.* **260,** 6481–6487.
19. Calzada-Garcia, J. A., Perez Gonzalez, M. N., Benito-Andres,

F. J., Morales Garcia, L. J., Cabezas, J. A., and Sanchez-Bernal, C. (1996) *Mech. Ageing Dev.* **87,** 1–13.

20. Hutchinson, W. L., A, I. L., Saraiva, M. J., and Pepys, M. B. (1996) *Neuromusc. Disord.* **6**(S), S16.
21. Clark, S., Muggli, E., La Greca, N., and Dunlop, M. E. (1995) *Diabetologia* **38,** 1131–1137.
22. Muriel, P., Ostoa-Saloma, P. U., Reyes-Esparsa, J. A., and Rodriguez-Fragoso, L. (1997) *J. Appl. Toxicol.* **17,** 145–151.
23. Poznanovic, G., Grujic, V., Ivanovic-Matic, S., and Sekularac, S. (1996) *Cell Biol. Intern.* **20,** 751–762.
24. Sundelin, J., Laurent, B. C., Anundi, H., Tragardh, L., Larhammar, D., Bjorck, L., Eriksson, U., Akerstrom, B., Jones, A., and Newcomer, M. (1985) *J. Biol. Chem.* **260,** 6472–6480.