Vol. 158, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1989 Pages 341-347

TRANSTHYRETIN MICROHETEROGENEITY AND THYROXINE BINDING ARE INFLUENCED BY NON-AMINO ACID COMPONENTS AND GLUTATHIONE CONSTITUENTS

Tom M. Pettersson[1,*] Anders Carlström[1], Anders Ehrenberg[2], and Hans Jörnvall[3]

[1] Department of Clinical Chemistry, Danderyd Hospital, Karolinska Institutet, S-182 88 Danderyd, Sweden

[2] Department of Biophysics, University of Stockholm, S-106 91 Stockholm, Sweden

[3] Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, Sweden

Received December 8, 1988

Two non-amino acid components as well as the glutathione constituents in labile associations with transthyretin (TTR) have been detected by preparative polyacrylamide gel electrophoresis from preparations isolated by affinity chromatography on Sepharose-bound retinol-binding protein (RBP). Incubation of native or reduced TTR with these novel components influenced the quaternary structure and caused reactions with reduced TTR in particular. Reduction of isolated TTR monomers released cysteine from the quantitatively major monomer, but non-amino-acid components from another dominating monomer. The reaction patterns also influence thyroxine (T4) binding. These relationships indicate that interactions in serum of TTR with constituents of glutathione and components different from T4 and retinol-RBP are important for the metabolism and function of TTR.

Transthyretin (TTR) or prealbumin is a multifunctional protein interacting with several molecules of biological interest. The complex with the retinol binding protein (RBP) has been demonstrated in several animal species [1]. The thyroid hormone binding property has also been well studied [2]. The relative importance of thyroxine-binding globulin (TBG) and TTR, respectively in thyroxine (T4) binding is not established in general. However, TTR in humans under physiological conditions is of almost the same importance as TBG in this respect [3]. Overall, TTR is more widespread than TBG and has been demonstrated in 15 vertebrate species, but TBG only in larger mammals [1].

TTR is involved in familial amyloidotic polyneuropathy [4] but also in senile amyloidosis [5]. In the hereditary form, amino acid substitutions in TTR are believed to cause the disease but in the senile form the reason for TTR deposits is unknown. Human TTR activates the immune response and this activity is connected with the N-terminal domain [6].

* Corresponding author.

0006-291X/89 $1.50

A complex microheterogeneity has been demonstrated for human TTR [7]. The lability of the quaternary structure and the condition of the Cys residue present in each monomer explain part of the TTR microheterogeneity [7]. Some serum factor enhances the dissociation of human TTR into monomers [7]. Inhibitors of thyroxine-binding to TTR apparently also exist in human serum [3]. The human TTR-T4 complex has been demonstrated to facilitate the bio-availability of T4 in rat by mechanisms of enhanced dissociation of T4 from the protein [8].

In this work, we demonstrate the presence of two hitherto unknown components and of the glutathione amino acid constituents in native preparations of TTR. These complexes influence the quaternary structure of TTR and therefore T4-binding.

MATERIALS AND METHODS

Preparations. RBP and TTR were isolated from serum by sequential affinity chromatography on Sepharose-human TTR and Sepharose-human RBP [7]. The TTR preparation was concentrated by freeze drying in order to retain associated molecules. About 30 l serum was used for one preparation. Preparative polyacrylamide gel electrophoresis was performed as reported [7] with the columns pre-run before application of the TTR sample to avoid contaminants from the polyacrylamide. The small-molecular fraction concentrated by freeze-drying and the protein fraction were desalted on Sephadex G-15 (1.6x50cm) and Sephadex G-25 fine (1.6x40cm), respectively. The small-molecular fraction was separated into a methanol-fraction and a water-fraction by gentle mixing in water, followed by solubilization in methanol of the water-insoluble remainder. TTR consisting of homogeneous monomers (M1,M2 and M3 as defined in [7], cf Fig. 3) was isolated [7] and reduced (50 mM ß-mercaptoethanol under N_2). Molecules released upon reduction were isolated on Sephadex G-25 fine in water.

HPLC. The methanol fraction was examined by C18 HPLC with 100, 98, 95, 90 or 80% methanol in water. The water fraction was examined by anion exchange HPLC (Nucleosil SB) with a formate gradient from 0.1 to 1.0 M, pH 2.8, or by counter-ion C18 HPLC in 30% methanol, 69% water, 1% HAc and 2.5 mM tetrabutyl ammonium iodide. The small-molecular fraction released from TTR upon reduction was examined by C18 HPLC with a gradient from 0 to 90% methanol in water. Absorbancies were monitored at 280, 254 and 220 nm.

Analytical procedures. The techniques utilized at different stages of the work are outlined in Fig. 1. The lability of the complexes in the small-molecular weight fraction from the polyacrylamide gel electrophoresis was analysed by gel filtration on Sephadex G-15 (1.6x150cm) in water. The isoelectric focusing was performed in 80% Iso Gel agarose (Marine Colloids) and 20% agarose Z (Pharmacia) as reported [7] under non-denaturating, denaturating (7 M urea, BDH Aristar), or both denaturating and reductive (by addition of 50 mM ß-mercaptoethanol or dithiothreitol) conditions. Separate effects of denaturation and reduction of native TTR were observed in two-dimensional experiments (cf Fig 3) performed as reported [7]. Amino acid analysis was carried out with a Beckman 121 M analyzer. UV spectra were obtained on a (Hitachi) double-beam spectrophotometer.

RESULTS

Composition of the small-molecular fraction associated with TTR. The TTR preparation obtained after affinity chromatography on Sepharose-human RBP was essentially pure (> 98%). However, it contained small-molecular weight components (fraction **I** in Fig. 1) that were eluted ahead of the main TTR fraction upon polyacrylamide gel electrophoresis. The desalted pre-fraction had a yellow color (absorbance maximum around 425 nm) as did also the TTR-fraction. The small-molecular fraction separated into three fractions (Fig. 1) upon gel filtration on Sephadex G-15 in water. The longer the column, the more material was shifted into peaks representing smaller complexes. By gentle mixing of the freeze-dried fraction with water, water-soluble components were extracted and

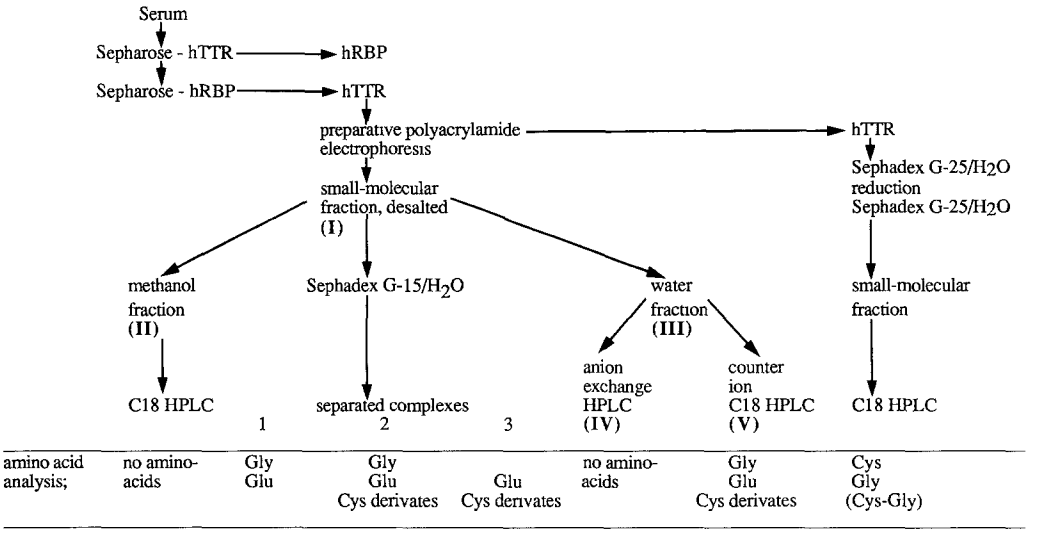

Figure 1. Scheme of the different isolation steps utilized, and the presence of amino acids associated with TTR.
Fraction I represents the total small-molecular weight fraction, fraction II contains the yellow component, fraction IV the colorless components and fraction V represents the fraction that contains essentially only amino acids. Fractions 1, 2 and 3 are eluted in that order from the Sephadex G-15 column.

the remainder was dissolved in methanol. The methanol fraction (fraction **II** in Fig. 1) was strongly yellow with an absorbance maximum around 425 nm and tailing up to 550 nm. The yellow fraction was homogeneous on C18 HPLC. The colored component is not a retinoid, flavonoid or bilirubin. From the water fraction (fraction **III** in Fig.1), two additional components were isolated to homogeneity upon anion exchange HPLC. They were interconvertable upon rechromatography. These components are colorless (fraction **IV** in Fig. 1). Upon counter-ion C18 HPLC, the water fraction separated into two peaks, one contained essentially the colorless components and the other (fraction **V** in Fig. 1) essentially amino acids. All fractions were submitted to amino acid analysis. Glycine, glutamic acid, and derivates of cysteine, including cysteic acid, were detected as shown in Fig.1. Analysis of the fractions without previous hydrolysis demonstrated un changed amino acid compositions. Consequently, the free amino acids representing the constituents of glutathione are associated with the yellow and the colorless components bound to TTR.

Reactions of components of the small-molecular fraction with TTR. The fractions, **I, II** and **V** (Fig. 1) were incubated with native and reduced TTR (Fig. 2). Fraction **V** reacts with the native TTR and gives rise to a protein band on the anodal side of subunit M2 (defined as in [7]). The same fraction reacts also with reduced TTR, resulting in two additional protein bands, one corresponding to M3 and one to a form between M1 and M3. Reaction with fraction **I** gives some material in the latter position. Additionally, both fractions **I** and **V** enhance the dissociation of the TTR tetramer into subunits as seen from the isoelectric focusing in the presence of urea (Fig. 2). The yellow fraction (**II**) does not at all affect the microheterogeneity pattern.

Components released from TTR upon reduction. Reduction of pure TTR preparations release nine components separable upon C18 HPLC. The glutathione-constituents cysteine, cysteinyl-glycine

and glycine were identified. Cysteine was deduced to constitute the main fraction and was isolated in a disulfide with ß-mercaptoethanol as identified by NMR spectroscopy. Homogeneous TTR tetramers consisting of monomers M1, M2 or M3 were separately studied, and the amino acids were demonstrated to be associated only with monomer M1 (at least 30% of this monomer carried a cysteine adduct). No amino acids were released from M2 upon reduction, but some unidentified complexing components as judged from the Sephadex G-25 elution pattern.

Effect of reduction on the TTR microheterogeneity. Non-denaturating isoelectric focusing in two dimensions demonstrates that native TTR tetramers are unstable as shown by conversions (Fig. 3A). Two-dimensional denaturating isoelectric focusing demonstrates spontaneous conversions between monomers which indicate release of a non-covalently bound component from the M2 monomer in particular (Fig. 3B). Introduction of a reducing agent into the denaturating isoelectric focusing of the second dimension converts a minor fraction anodal of M2 into the position of M3, some material anodal of M1 into a position between M1 and M3, and potentiates the spontaneous conversion of M2 to M1 (Fig. 3C).

Two groups of monomers that behave differently as observed by denaturating isoelectric focusing followed by SDS/PAGE. Four minor monomeric fractions (indicated by arrows 1 to 4 in Fig. 3B) behave differently from the major monomeric forms M1 and M2. The minor fractions are more resistant to SDS denaturation and do not easily reassemble the tetrameric configuration upon renaturation from urea. M3 is one of these more rigid TTR monomers. The concentration of this monomer was also increased upon reaction of reduced TTR with fraction V (Fig. 2).

Conclusion. A yellow colored component, a colorless component and the glutathione-constituents Gly, Glu and Cys are present in serum in associations with TTR. These components react specifically with reduced TTR, influence the TTR microheterogeneity, modulate the stability of TTR monomers and the dynamic behavior of TTR tetramers. The influence on the quaternary structure of TTR may be of physiological significance as dissociation into monomers releases T4 bound to TTR and thereby strongly influences the plasma concentration of free hormone.

DISCUSSION

Small-molecular weight fractions that separate from TTR preparations isolated from stored and consequently oxidized serum pools contain, besides glycine and glutamic acid, predominantly cysteic acid among the detected cysteine derivates. The yellow fraction could not be separated from these preparations and the complexes did not separate in the same manner on Sephadex G-15 gel filtration as preparations from fresh serum. Thus, the extent of oxidation and the quantitative ratios between the yellow component, the colorless component and the amino acids are essential for the behavior of the complex.

Pure TTR preparations are also yellow absorbing in the same range as the isolated yellow fraction. From TTR preparations isolated by phenol precipitation, the yellow component can be extracted by acidic methanol. The release of non-amino acid components from monomer M2 upon reduction, conversions of TTR tetramers under native conditions (cf Fig. 3A), and conversions of TTR monomers under denaturating conditions (cf Fig. 3B), indicate non-covalent association to TTR of components that are not related to thyroxine or retinol-RBP. Thus, all three components (amino

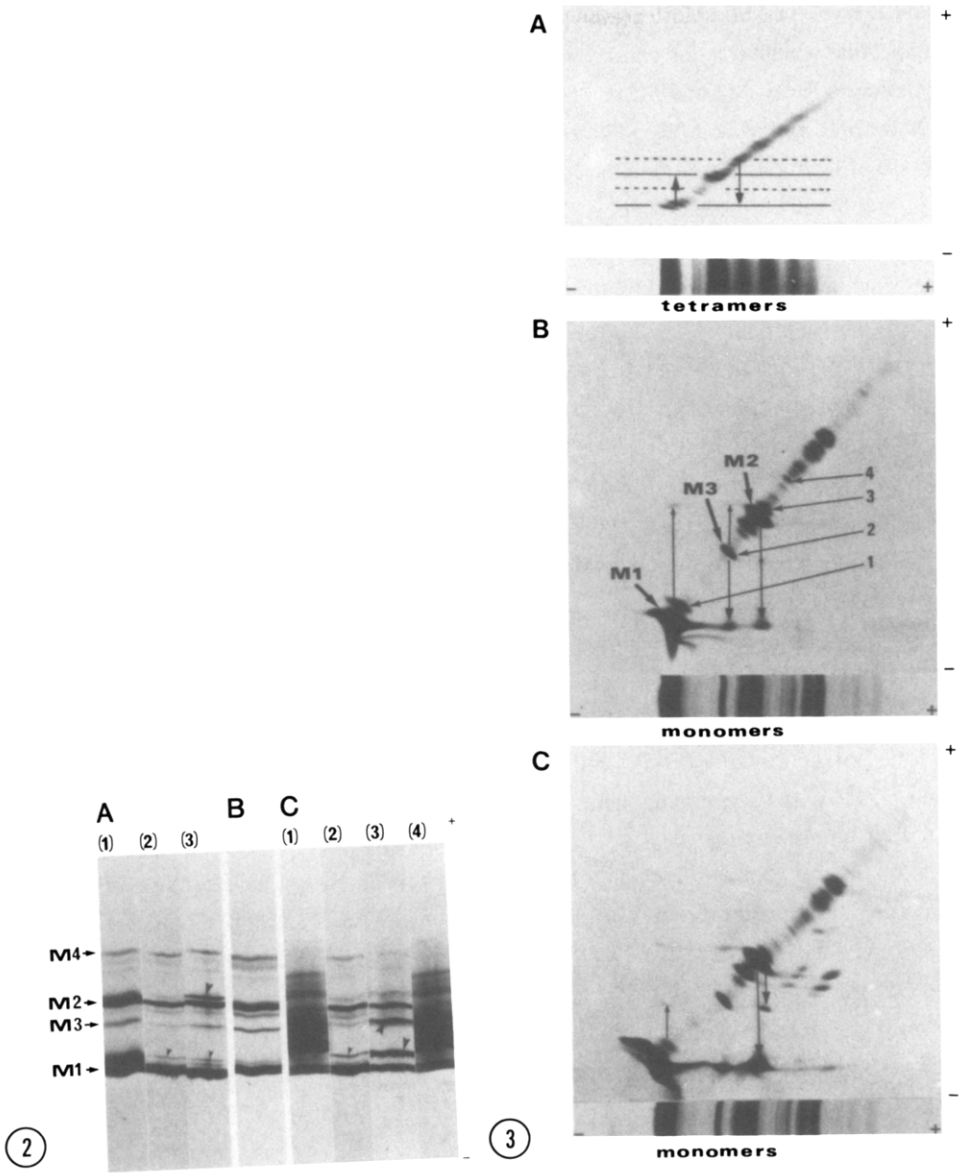

Figure 2. Denaturating (7M urea) isoelectric focusing of native TTR in A (focusing time 3 h); native TTR in B (focusing time 5 h) and reduced TTR in C (focusing time 3 h). A and C after no pretreatment (1) after incubation with total small-molecular weight fraction (2), with the fraction containing essentially the amino acids isolated upon counter ion C18 HPLC (3), or with the yellow fraction (4). The three main changes in the TTR microheterogeneity pattern are indicated by arrows.

Figure 3. Spontaneous conversions between TTR tetramers (A) and monomers (B), as well as conversions between TTR monomers caused by reduction (C) observed by two dimensional isoelectric focusing. Conversions are indicated by vertical arrows. A, non-denaturating and non-reducing conditions in both dimensions; B, denaturating conditions in both dimensions; C, denaturating condition in the first dimension and denaturating plus reducing conditions in the second. The minor monomeric forms that are more SDS resistant, less susceptible to tetramer reassembly, and formed by reaction with TTR associated components now reported, are indicated by arrows 1 to 4.

acids and the yellow and colorless non-amino acid components) isolated in the TTR-associated small-molecular weight fraction can separately be isolated from pure TTR.

The monomeric forms M1 and M2 are heterogeneous and can each be separated into three forms (M1a, M1b, M1c and M2a, M2b, M2c, respectively) in the presence of serum [7]. In fresh serum, about 40 % of the monomers are in the M1b form, with an intact reactive thiol. In M2b the thiol is oxidized, and M2a is considered to be a glutathione adduct. In TTR preparations, M1a and M2b are the dominating monomers, and M1a, as shown here, the cysteine adduct. Thus, the microheterogeneity is influenced by the components of the small-molecular fraction as presently reported.

In the serum environment, the reactive thiol of the M1b monomer is protected by association with the retinol-RBP complex [7]. Components of the small-molecular weight fraction react preferentially with reduced TTR. The binding of T4 to TTR under physiological conditions averages 44% of total T4 [3]. For the binding of T4 to the binding site located deep in the TTR tetramer the protein structure must be dynamic [2]. The isolated components influence the stability of the TTR tetramer. Thus, the components reported here are indirectly dependent on RBP for reaction with TTR and affect T4 binding upon association to TTR.

TTR deposits in familial amyloidotic polyneuropathy are connected with discrete amino acid substitutions [4], while the involvment of normal TTR in senile amyloidosis indicates that other metabolic factors can also be involved. T4 binding to the TTR in two variants of familial amyloidotic polyneuropathy (FAP I and II) is reduced [9]. In this context, the association and reaction of TTR with serum components as presently reported is of importance. The stimulatory effect of TTR on the immune response [10] may also depend on these relationships as native TTR has about 60 times higher activity than the isolated N-terminal, 10-residue fragment [6].

In summary, the serum components in labile associations with TTR as now reported react with the TTR monomer and influence the dynamic behavior of the TTR tetramer. Besides the direct influence on the T4-binding of TTR and thereby the concentration of free T4 in plasma, these relationships integrate the three functional fields connected with TTR, i. e. vitamin A metabolism, thyroid metabolism, and immune response.

ACKNOWLEDGEMENTS

This project was supported by grants from the Swedish Medical Research Council (projects 03X-3532 and 6231).

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