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Amyloidogenic properties of transthyretin-like protein (TLP) from *Escherichia coli*

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Abstract **We report the amyloid-like properties of *Escherichia coli* transthyretin-like protein (TLP). TLP is 32% homologous to human transthyretin (hTTR), and is also tetrameric. In contrast to hTTR, TLP does not bind thyroxine. TLP orthologues are found in several prokaryotes, lower eukaryotes and vertebrates. TLP carries a signal peptide that targets the protein to the periplasmic space. We found that TLP and hTTR tetramers dissociate into monomers under similar conditions, although TLP monomers have different association properties. Like hTTR, TLP forms aggregates, small fibrillar structures of 8 nm width, and annular structures of 8 nm diameter which present amyloid-like properties and are toxic to cells.**

Keywords: Transthyretin; Transthyretin-related protein family; Evolution; Amyloid; Protein aggregation; Cell toxicity; *Escherichia coli*

1. Introduction

In almost all vertebrates, transthyretin (TTR) is responsible for the transport of retinol binding protein in complex with retinol (vitamin A) and of thyroid hormones – thyroxine (T4) and 3,3′,5-triiodothyronine (T3). TTR evolved in the choroid plexus of stem reptiles 350 million years ago [1]. Liver TTR synthesis appears to have originated independently much later, about 50 million years ago, in the lineage leading to today's eutherians, diprotodont marsupials, polyprotodont marsupials, birds and fish [2]. The overall three-dimensional structure of vertebrate TTR has not changed significantly during evolution [3]. Amino acid differences between species lie on the surface of the molecule. Most changes are located within the first 10 amino acids from the NH_2-terminus. There has been a successive shortening of the 5′ end of exon 2, resulting in a shorter and more hydrophilic N-terminal region [4].

The central channel that harbours the thyroid hormone-binding site is highly conserved in all TTRs [5]. Although structure has been conserved, the functional properties of TTR have changed during evolution. The main differences include tissues of synthesis (liver and/or choroid plexus) and the decrease of T3 affinity in favour of T4 [6].

An increasing number of prokaryotes and lower eukaryotes with sequences homologous to TTR, the transthyretin-like proteins (TLPs), are emerging since their first mention in 1997 by Sonnhammer and Durbin [7] and later in 2000 by Monaco [8] and Prapunpoj et al. [9].

It has been suggested that TLP could be an ancestor of TTR [3,10] that arose from a duplication event [11]. Organisms with a characterized function for TLP protein include: *Arabidopsis thaliana* [12]; *Bacillus subtilis* [13,14]; *Escherichia coli* [15] and *Salmonella dublin* [16]. TLP from *A. thaliana* was identified as a membrane associated protein involved in control of growth through association with the brassinosteroid-insensitive 1 receptor (BRI1) [12]. *Caenorhabditis elegans* has two TLP genes [17]; both genes are transcriptionally regulated during development. However, inactivation of TLP expression by double-stranded (ds) RNA interference yielded no phenotype. In *B. subtilis*, however, TLP inactivation resulted in an uricase-defective phenotype [13] leading to the proposal that TLP was responsible for the hydrolysis of 5-hydroxyisourate. A similar role in uric acid metabolism was found for TLP from *E. coli* [15], *S. dublin* [16] and *Mus musculus* [18].

Eneqvist et al. [17] characterized TLPs from *E. coli* and *C. elegans*. They showed that recombinant TLPs formed tetramers, as does TTR from vertebrates [11]. TLP from *E. coli* was reported to be a homotetramer of approximately 52 kDa. In contrast to hTTR, TLP from these organisms did not bind T4. Furthermore, partial acid denaturation, which converts hTTR to aggregates and fibrils with amyloid-like properties, had no effect on *E. coli* TLP [17].

In this work, we present studies performed with *E. coli* TLP that reveal its amyloidogenic potential and its toxicity in cellular studies.

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Abbreviations: TLP, transthyretin-like protein; TTR, transthyretin; TEM, transmission electron microscopy; Th-T, thioflavin-T

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2. Results

2.1. Physical–chemical characterization of TLP

To study its biochemical and physical properties, *E. coli* TLP protein or a FLAG peptide tag derivative was over-expressed and purified. TLP was analysed by gel filtration. A protein peak was identified that eluted similarly to homotetrameric hTTR, just ahead of the molecular weight calibration standard, ovalbumin (43 kDa) (Fig. 1A). This elution pattern supports a previous report that TLP in its native state is a homotetrameric protein [17]. The protein sequence of TLP was verified by mass spectrometry (see Section 4).

We then compared the dissociation of hTTR and untagged TLP tetramers. After boiling with or without β-mercaptoethanol, TLP migrated in SDS–PAGE as a monomer of approximately 16 kDa (Fig. 1B, lane 1 and data not shown). Non-heated samples contained both tetramers and monomers (Fig. 1B, lane 2) and treatment with β-mercaptoethanol did not affect the monomer/tetramer ratio (not shown). Heated hTTR dissociated mostly to a monomer, although some dimeric species persisted (Fig. 1B, lane 3). Non-heated hTTR migrated as a dimer (Fig. 1B, lane 4); treatment with β-mercaptoethanol did not dissociate dimeric hTTR (not shown).

These results indicate that hTTR dimer is more stable than the TLP dimer. This instability was seen for both untagged TLP and the FLAG derivative.

2.2. Amyloidogenic properties

hTTR is able to form fibrils in vitro upon acidification [19,20]. At low pH, hTTR tetramer dissociates into structurally modified monomers, favouring aggregation and fibril formation. hTTR fibrils are mostly 8 nm wide, although 4–5 and 9–10 nm wide fibrils can also be observed. With extended incubation at low pH, hTTR fibrils increase to 300 nm in length.

Sequence and structural similarities between TLP and hTTR suggested that the ability to aggregate and form fibrils might be conserved between the two proteins. We therefore asked if TLP could form amyloid under conditions other than those used by Eneqvist et al. [17]. Consistent with previous reports, TLP acidification did not result in the thioflavin-T (Th-T) binding characteristic of an amyloid fold (not shown; [17]). A different protocol for TLP fibril formation was then tested, which entailed incubating the protein at various pH values at 24 °C with stirring (Fig. 2A, chart). At low pH (3.0 or 3.6) no amyloid was formed. At higher pH values, however, (pH 4.8–6.8) an increase in fluorescence intensity was observed after Th-T addition, consistent with formation of amyloidogenic TLP. Similar results were obtained with FLAG-tagged TLP, i.e., amyloid formation was dependent on stirring at pH 4.8–6.8. Thus, for convenience, we used FLAG-tagged TLP in the following experiments to demonstrate and characterize amyloid formation. TLP was incubated for 3 days at 24 °C with stirring at pH 5.8 and then examined with cross polarized light microscopy after Congo red binding

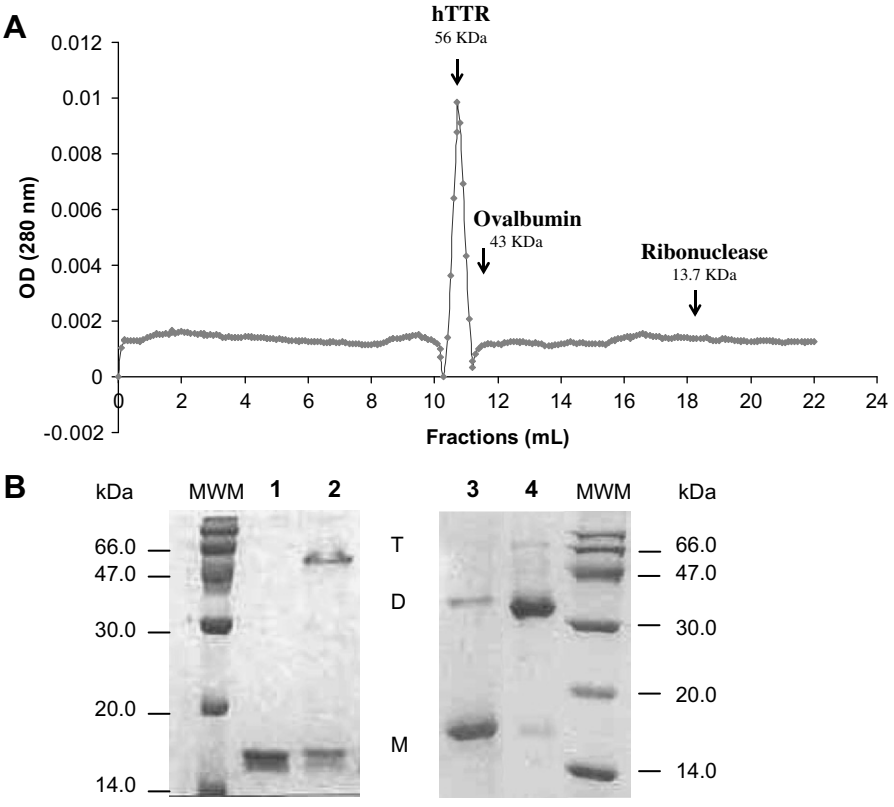

Fig. 1. (A) TLP homotetramer. TLP analysed by gel filtration shows a peak that eluted close to hTTR (56 kDa) and before protein calibration standards ovalbumin (43 kDa) and ribonuclease A (13.7 kDa). (B) Tetramer stability under denaturing and reducing conditions. SDS–PAGE of TLP and hTTR proteins. Lane 1: TLP boiled with β-mercaptoethanol; lane 2: TLP not boiled, with β-mercaptoethanol; lane 3: hTTR boiled with β-mercaptoethanol; lane 4: hTTR not boiled, with β-mercaptoethanol. Omission of β-mercaptoethanol gave similar results (not shown). T – tetramer; D – dimer; M – monomer.