Short Conceptual Overview

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Transthyretin microheterogeneity and molecular interactions: implications for amyloid formation

Abstract: Aggregation of transthyretin (TTR), a plasma-binding protein for thyroxine and retinol-binding protein, is the cause of several amyloid diseases. Disease-associated mutations are well known, but wild-type TTR is, to a lesser extent, also amyloidogenic. Monomerization, not oligomer formation as in several other depository diseases, is the rate-limiting step in TTR aggregation, and stabilization of the natively tetrameric form can inhibit amyloid formation. Modifications on Cys10, as well as interactions with native ligands in plasma, were early found to influence the equilibrium between tetrameric and monomeric TTR by dissociating or stabilizing the tetramer. Following these discoveries, synthetic ligands for pharmacological prevention of TTR aggregation could be developed. In this article, we outline how the different types of TTR interactions and its microheterogeneity in plasma are related to its propensity to form amyloid fibrils. We conclude that plasma constituents and dietary components may act as natural TTR stabilizers whose mechanisms of action provide cues for the amelioration of TTR amyloid disease.

Keywords: amyloid disease; drug design; ligand binding; plasma components; protein dynamics.

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Abbreviations: EGCG, epigallocatechin 3-gallate; FAC, familial amyloid cardiomyopathy; FAP, familial amyloid polyneuropathy; GSH, glutathione; RBP, retinol-binding protein 4; SSA, systemic senile amyloidosis; TTR, transthyretin; T4, thyroxine; WT, wild type.

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Introduction

Transthyretin (TTR, initially known as prealbumin) is predominantly a carrier protein, mainly associated with the transport of thyroxine (T4) and the complex of retinol and retinol-binding protein 4 (RBP) in plasma (1). Emerging evidence indicates a complex role of TTR in the distribution of T4 and retinol (2). Besides its transporter functions, TTR is implicated in a wide variety of additional physiological processes, such as thermoregulation and endocrine roles, and is produced at several sites. In addition, it has been shown to exert protease activity and chaperone-like actions (3, 4), which may provide links between TTR and Alzheimer's disease (5, 6).

Under physiological conditions, as prevailing in the circulation, TTR predominantly exists as tetramers composed of two dimers of the 127-residue monomer. The molecular architecture of the TTR tetramer has been studied extensively (7), yielding atomic-resolution information about ligand interactions (8), the architecture of the retinol transport complex with RBP (9), and the effects of mutations on the amyloidogenic propensities of TTR (7, 10). Presently, pharmacological intervention and drug development efforts are promising approaches to treat TTR amyloidosis (11, 12).

In early experiments, our group identified sequence similarities among TTR, RBP, and gastrointestinal hormones, indicating potential interactions (13). Already then, purification of native TTR from sera suggested a monomer-dimer-tetramer equilibrium and ligand interactions that are influenced by factors in native plasma and by protein modifications (14–17). Those early findings were of unclear significance at the time but have hinted at the complex relationship among TTR modifications, oligomerization, and ligand interactions. In this article, we survey recent insights into the relationships of old observations and present conclusions about possible roles of plasma factors in both TTR aggregation and protein dynamics.

TTR folding and amyloid formation

TTR can cause amyloid diseases (18), ranging from sporadic deposits in the heart and blood vessels of aged individuals (systemic senile amyloidosis, or SSA) to severe systemic or local amyloidoses with polyneuropathy (familial amyloid polyneuropathy, or FAP) or cardiac myopathy (familial amyloid cardiomyopathy, or FAC) (19). The overwhelming majority of the TTR-related depositary diseases are caused by mutations that alter the stability of its tertiary or quaternary structure, and in this manner, promote amyloid formation (see below). However, wild-type (WT) TTR also displays a considerable aggregation propensity and has been identified as the major component of amyloid deposits in SSA (20). Studies of *ex vivo* preparations of protein deposits from patients with sporadic and familial forms of TTR-derived amyloidosis have uncovered that proteolytically cleaved TTR is present in distinct forms of amyloid fibrils (21–24). In the case of the S52P mutant, these fragments were found to be significantly more amyloidogenic than the corresponding full-length TTR and therefore possibly act as triggers for aggregate formation (23).

Several lines of evidence point to the full-length TTR monomer as the amyloidogenic species and its detachment from the native TTR tetramer as the rate-limiting step for fibrillogenesis (25). Overall, monomerization as requirement for fibrillation is unusual among amyloidogenic proteins because oligomerization is otherwise often the first step in protein aggregation. Pioneering work on recombinant TTR by the group of Jeffery Kelly has demonstrated that the WT TTR tetramer is significantly destabilized when exposed to a mildly acidic environment (26). Crystallographic analyses revealed that the only helical portion of TTR, the EF-loop-helix-region, is partially disordered in two of the four TTR subunits in response to low pH, which leads to reduced contacts between the two halves of the tetramer (27). As a result, the native TTR tetramer dissociates more readily into aggregation-competent monomers. However, changes in the quaternary structure alone are not sufficient to trigger amyloid formation but merely lead to a loss of protection against the factors that initiate aggregation (28).

The subsequent aggregation of the monomer into amyloid fibrils occurs *via* additional unfolding events that could even prevent tetramer reassembly (29). NMR studies on an engineered TTR monomer with WT-like structure suggests that this conversion is a stochastic process facilitated by localized structural fluctuations (30). It is, however, impacted by a wide variety of natural factors, as outlined below.

Mutations link TTR stability and amyloid disease

Naturally occurring point mutations in TTR provide a direct link between protein stability and amyloid disease. Over 100 TTR mutations that cause familial forms of TTR amyloidosis have been identified to date (www.amyloidosismutations.com/attr). Mapping of the most prevalent mutations in the tetramer structure and the analysis of their effects on the TTR stability *in vitro* have provided further clues to the molecular underpinnings of TTR aggregation (10). It should, however, be noted that the change in stability conveyed by a specific mutation does not alone correlate with its pathogenicity *in vivo* because of cellular protection mechanisms that prevent some highly aggregation-prone variants from being secreted (31).

The disease-associated mutations promote aggregation by destabilizing the quaternary structure of the tetramer or the tertiary structure of the subunits. This is exemplified by three point mutations that have yielded detailed information about the relationship between the molecular contacts in the tetramer and the tendency to form amyloid deposits. The L55P mutation gives rise to early-onset FAP (32), increases the subunit exchange rate of the tetramer compared with the WT form, and leads to more frequent release of monomeric TTR, which can then aggregate (10). The V30M mutation, which is also associated with FAP (33), does not affect tetramer stability but increases the aggregation propensity of the released monomer (10). The T119M mutation, meanwhile, stabilizes the tetrameric conformation by providing additional contacts at the dimer-dimer interface (10). It also delays onset of cerebrovascular disease and prolongs life expectancy (34). Hammarström et al. (35) were able to show that coexpression of V30M and T119M prevents TTR fibrillation by locking the aggregation-prone V30M monomers into stable heterotetramers with T119M subunits, demonstrating that tetramer stabilization can ameliorate TTR-related amyloidosis.

Cysteine modifications have differential effects on TTR aggregation

The frequent occurrence of SSA suggests that age-related modifications, specifically protein oxidation, may affect the aggregation propensity of TTR and promote amyloid disease (36, 37). Early electrophoretic studies showed

multiple isoelectric points and sizes for plasma TTR, which has also been attributed to the presence of protein modifications (14) including different oxidation states (38) of the cysteine residue at position 10. Further investigations then led to the discovery that Cys10 can form mixed disulfides with glutathione (GSH) and its fragments or with other S-reactive components (14, 15). TTR tetramers purified from plasma were found to contain different combinations of possible modifications at this position, and Cys10-modified TTR monomers could be reassembled into mixed tetramers (14, 15). Interestingly, these thiol group alterations were found to affect the monomer-tetramer equilibrium (14, 15).

In line with this conclusion, modification of Cys10 with cysteine, full-length GSH, and CysGly fragments were found to destabilize WT TTR and the V30M variant *in vitro* (39, 40). Although it was reported that the presence of mixed disulfides predominantly affects the aggregation propensity of monomeric TTR at mildly acidic pH (39), analytical ultracentrifugation experiments have shown that *S*-cysteinylation also enhances tetramer dissociation at physiological pH (41). The heterogeneous oxidative modifications produced by carbonylation of TTR destabilize its monomeric form and interfere with the binding of tetramer-stabilizing compounds (42).

Interestingly, the oxidized Cys10 variant, which has also been found in plasma preparations of TTR, inhibits aggregation, and it has been speculated that this is due to an increased tetramer stability of this form (43). Therefore, oxidation-related or other modifications of Cys10 all affect the aggregation propensity and tetramer stability of TTR, but to different extents and in multiple manners, underlining the importance of protein microheterogeneity in amyloidoses.

S-Sulfonation of Cys10 was identified as another naturally occurring modification with differential effects on TTR stability (Figure 1A). Cysteine *S*-sulfonation was observed in patients suffering from hereditary amyloidosis. However, this occurred in connection with the introduction of a second cysteine, also *S*-sulfonated, through a point mutation at position 33. Hence, the altered aggregation propensity of the *S*-sulfonated TTR could in this case not be ascribed solely to the sulfonation (44). Instead, several studies have identified that *S*-sulfonation of WT and V30M TTR increases the stability of the tetrameric form by providing additional intramonomeric contacts and in this manner prevents amyloid formation (41, 45, 46). This raises the possibility that increased *S*-sulfonation of Cys10, for example, through the dietary supplementation of sulfites, may have a protective effect against TTR amyloidosis (47).

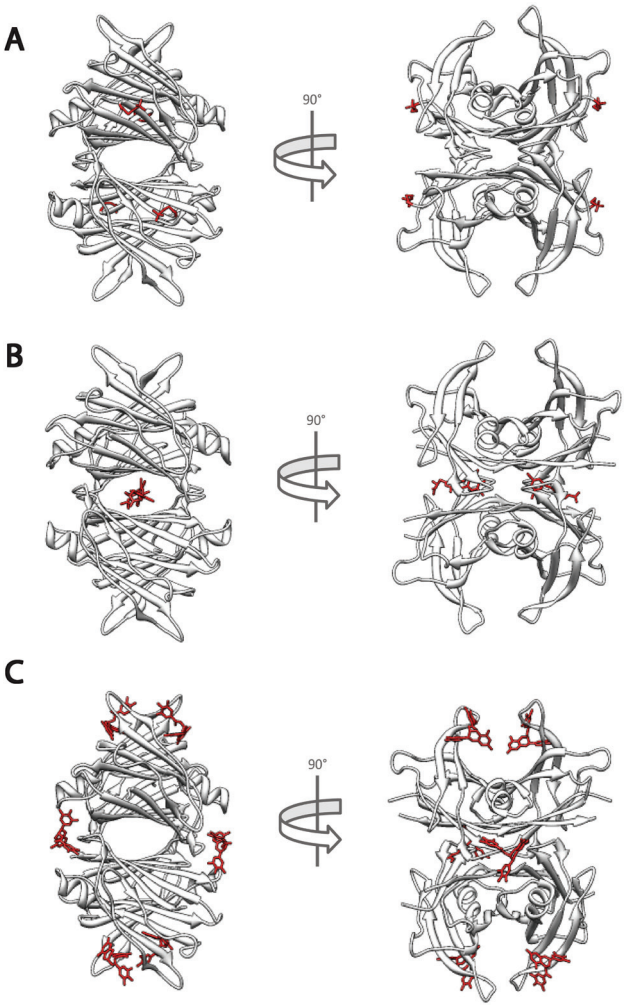

Figure 1 Examples of interaction sites that can be targeted to prevent TTR aggregation.
(A) Cys10 sulfonation (red) stabilizes intramolecular interactions and reduces monomer unfolding (PDB ID 2H4E). (B) T4 binding (red) to the hydrophobic channel between the subunits reduces tetramer dissociation by increasing inter-subunit contacts (PDB ID 1ICT). (C) EGCG binding (red) supports the otherwise labile tetrameric form of the V30M mutant (PDB ID 3NG5).

Interactions at the T4-binding pockets reduce tetramer dissociation

Another source of plasma TTR heterogeneity besides oxidative modifications was found to be the dynamic monomer/tetramer equilibrium of TTR, and its self-association may be affected by plasma components. For example, zinc can interact with tetrameric TTR *via* four low-affinity-binding sites and destabilize the monomeric form (48).

The discovery that TTR aggregation can be prevented by stabilization of its tetrameric form was quickly followed by reports that T4, the primary TTR ligand, inhibits TTR aggregation (49). The two T4-binding sites on the tetramer form two deep hydrophobic cavities that are connected at the ends. Both have a positively and a negatively charged residue at the entrance, which interact with the charges on the zwitterionic T4 ligand (8). Bound T4 acts as mediator for interactions between both halves of the tetramer and prevents the release of the aggregation-prone monomer (Figure 1B) (49).

However, only a small amount of the binding sites in plasma TTR tetramers are occupied (50–52), blurring the significance of its stabilizing effect *in vivo*. Nevertheless, the availability of TTR-binding sites has spawned successful efforts to design synthetic ligands that mimic the molecular contacts of T4 in the TTR tetramer, while not interfering with the T4 biological actions throughout the organism (11, 53). Of these, the ligand tafamidis is currently in phase III clinical trials for the treatment of FAP (54), and other molecular classes approved for human use in other contexts, including nonsteroidal anti-inflammatory drugs such as diclofenac and diflusinal and antibiotics such as tetracyclines, can effectively stabilize tetrameric TTR (12).

T4 binding exhibits negative cooperativity, with only a single binding site occupied by the ligand (11). Bivalent inhibitors that engage both sites at the same time have emerged as an alternative framework for aggregation inhibitor design, in addition to the ligands that are based on the T4 structure. Ligands composed of two bisaryl moieties connected by a hydrophobic 7- to 10-carbon linker can be incorporated during tetramer formation to trap free TTR in non-dissociable complexes (55). Further modification of the end groups and an increased alkyl linker length of 11 carbons have yielded palindromic ligands that can bind to preformed tetramers. Co-crystallization with these ligands confirms the occupation of the two T4-binding sites and indicates how they can be inserted into the 'hourglass' channel of the TTR tetramer (56).

Although their pharmacological suitability is not yet fully explored, such bivalent ligands may have advantages over monovalent aggregation inhibitors. A single ligand-binding event is sufficient to reduce TTR aggregation (57), but the occupation of both binding sites at the same time provides greater tetramer stabilization (55). In addition, bivalent ligands are unlikely to compete with T4 for interactions with other T4 carrier proteins or receptors. If, however, the microheterogeneity and dynamic behavior of plasma TTR is relevant for physiological functions, the massive stabilization of tetrameric TTR might have more widespread effects than previously thought, as it cannot be ruled out that monomeric TTR may also fulfill specific physiological functions.

It is well established that interactions with RBP can stabilize the tetrameric TTR structure and prevent aggregation. The TTR:*holo*RBP retinol transport complex composed of four TTR molecules, two RBP molecules, and two retinol moieties is resistant to pH-induced aggregation, which can be attributed to the extensive interaction network between the two halves of the TTR tetramer and the two RBP (7). In addition, a recent study has provided evidence that complex formation with T4 and RBP, and also with free retinoic acid, can efficiently inhibit TTR tetramer subunit exchange and prevent aggregation (58). In the TTR-RBP-retinol complex, the TTR tetramer may bind two RBP-retinol moieties *in vitro* (9). In the absence of RBP, retinoic acid was found to compete with T4 for binding to the TTR tetramer and prevent subunit exchange, whereas nonionic derivates did not bind to TTR or affect its tetramer stability (58). With support from crystallographic studies (59), these findings suggest that retinoic acid can insert into the T4-binding sites between the TTR dimers, interact with the charged amino acids at the entrance though its carboxyl group, and in this manner stabilize the tetrameric form.

These effects of retinol then raise further possibilities in relation to our early demonstration that TTR isolated from human and chicken plasma or amyloid deposits from humans copurified with a yellow component (15, 22). In human TTR, the yellow component was found to be related to pterin (15). In chicken TTR, this compound was identified as lutein, a carotene derivate that differs from β-carotene by just two additional hydroxyl groups (17) (Figure 2). Despite the fact that TTR does not appear to be a physiologically relevant lutein carrier in humans (60), it has to be noted that lutein and several other carotenoids are structurally related to retinoic acid. Considering the successful use of T4-binding site ligands to prevent TTR aggregation (12) as well as the stabilizing effects of retinoic acid (58) and the ability to associate with lutein (17), it appears likely that other naturally occurring molecules with a similar architecture can potentially stabilize the TTR tetramer when present at sufficiently high concentrations. Bearing in mind the interactions of TTR with lutein and free retinoic acid, plasma components with similar structural properties could have beneficial effects in TTR-related amyloidosis, even if they do not require TTR as a physiologically relevant carrier in plasma.

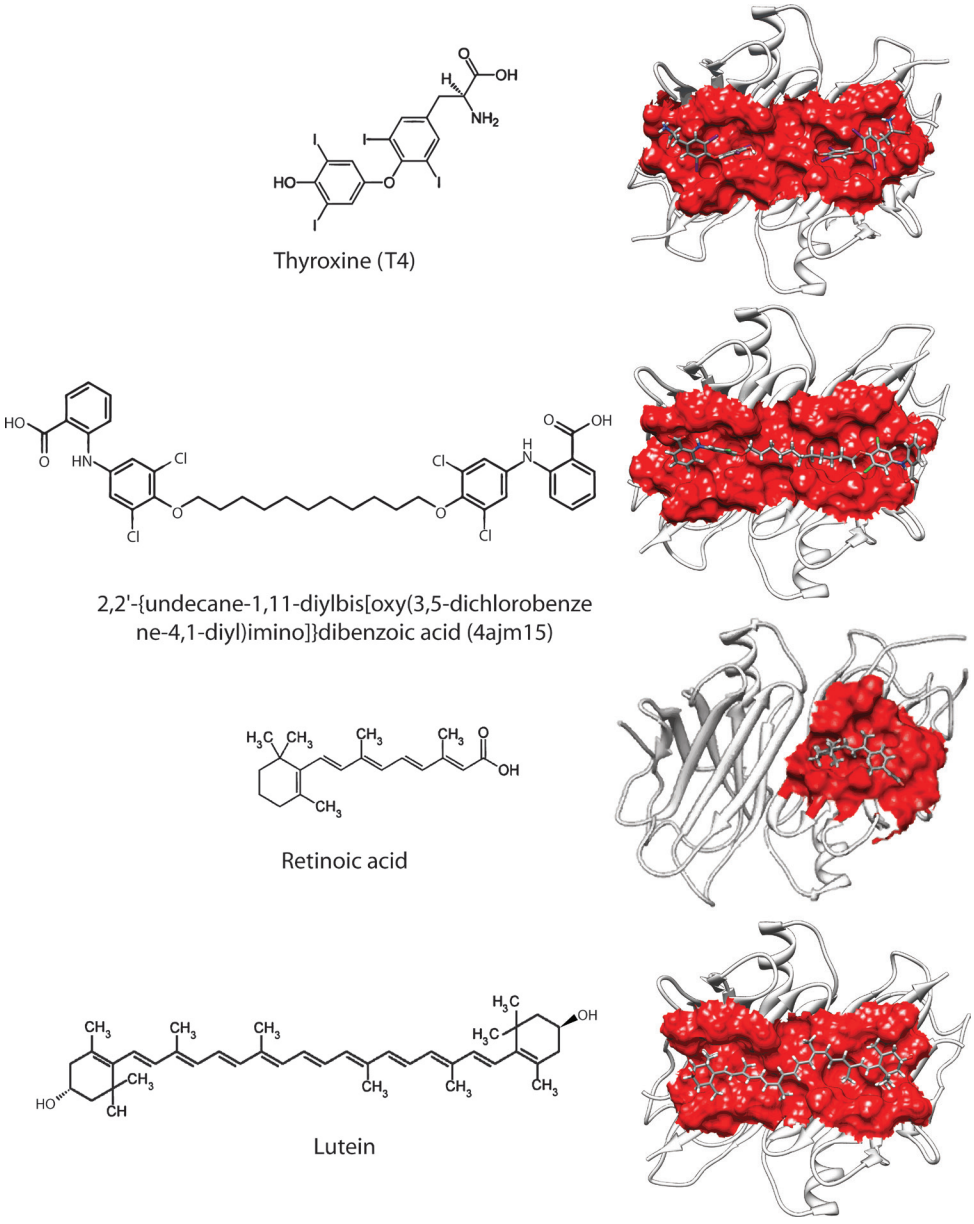

Figure 2 Experimental structures and computational models of TTR complexed with natural and engineered ligands bound to the T4 binding site(s).
Shown are surface renderings of the dimer-dimer interface of tetrameric TTR bound to (top to bottom): two T4 molecules (PDB ID 1ICT), the bivalent inhibitor 2,2′-{undecane-1,11-diylbis[oxy(3,5-dichlorbenzene-4,1-diyl)imino]}dibenzoic acid (PDB ID 3IPB), retinoic acid (PDB ID 1TYR), and, as a computational model based on PDB ID 3IPB, a lutein molecule fitted into the hydrophobic channel.

TTR can be stabilized though interactions at other sites

The inhibition of protein aggregation by other natural dietary components was previously established to ameliorate depository diseases. Examples are curcumin and the green tea polyphenol epigallocatechin 3-gallate (EGCG), both of which can inhibit the aggregation of Alzheimer's disease-associated Aβ peptide and Parkinson disease-associated α-synuclein (61, 62). Their activity against such a broad spectrum of amyloidogenic proteins suggests that these polyphenolic compounds interfere with the self-assembly of unfolded or refolded peptides rather than conserving their native conformations (61). However, it has been shown that EGCG prevents TTR aggregation by stabilization of the tetrameric conformation. X-ray crystallography revealed that EGCG binds to multiple sites on the

TTR tetramer, four of which are located at the interface between the dimers but exclude the T4-binding channel (Figure 1C) (63). Curcumin, meanwhile, was shown to interact with TTR tetramers specifically at the T4-binding site (64). The ingestion of either EGCG or curcumin alleviates amyloidosis in mouse models of FAP (64–66), and new evidence suggests that EGCG even halts the progression of FAC in humans (67).

The prevention of TTR aggregation without engaging the T4-binding channel can also be achieved through the use of high-affinity lysine-binding molecules termed molecular tweezers (68). Because lysine residues are key interaction points for several amyloidogenic proteins, molecular tweezers can prevent rearrangements of the protein structure and/or self-assembly of misfolded species (69). Although it is unclear which lysine positions and state(s) during TTR aggregation are targeted, molecular tweezers have been shown to effectively prevent amyloid formation *in vitro* and in mouse models of hereditary TTR amyloidosis (68, 70).

Trends and future directions

TTR amyloidosis may become an instance of a protein aggregation disease that can be ameliorated by pharmacological intervention. Although a close relationship between monomer formation and fibril formation has been uncovered, the factors that imbalance TTR stability *in vivo* are still unclear. Plasma contains monomeric, dimeric, and tetrameric TTR with modifications that have opposing effects on its aggregation propensity. The plasma components T4 and retinol as well as zinc ions can stabilize TTR but individually occupy only a small fraction of the available TTR-binding sites. The dietary administration of a wide range of compounds can be used as a strategy to stabilize the tetrameric form and prevent amyloid formation. In the light of the present-day findings with drugs and extrinsic compounds, a wider screen of the effects of previously discovered interaction partners like retinol and lutein, but also chemically active agents such as sulfites, might be motivated to evaluate combined effects on the TTR quaternary structure and its stabilization.

It is likely that TTR dynamics are controlled by a combination of factors. Transient binding of a multiplicity of plasma ligands may have a chaperone-like effect on monomeric or multimeric TTR or may lower the subunit exchange rates below the threshold for aggregation without a need for constant saturation. The ligand binding properties may also differ depending on the localization of the protein and stabilize TTR specifically under aggregation-promoting conditions. A similar example is the regulation of insulin solubility by proinsulin C-peptide, which has a monomerizing effect on insulin at granular pH (71). In the light of this, the molecular interaction networks of TTR in intracellular and extracellular environments may hold valuable information with regard to its roles in health and disease.

Highlights

- TTR is a plasma carrier protein for T4 and retinol *via* RBP4 and is associated with amyloid diseases.
- TTR aggregation is dependent on dissociation of the natively folded tetramer.
- Designed and natural TTR ligands can stabilize tetrameric TTR and prevent amyloid formation.
- Oxidative modifications and interactions with low molecular weight ligands affect the monomer-tetramer equilibrium in plasma.
- The microheterogeneity and the dynamic behavior of TTR controlled by different plasma factors might be related to its biological functions. Dietary influences are also possible.

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