Human transthyretin (TTR) is implicated in several fatal forms of amyloidosis. Many mutations of TTR have been identified; most of these are pathogenic, but some offer protective effects. The molecular basis underlying the vastly different fibrillation behaviours of these TTR mutants is poorly understood. Here, on the basis of neutron crystallography, native mass spectrometry and modelling studies, we propose a mechanism whereby TTR can form amyloid fibrils via a parallel equilibrium of partially unfolded species that proceeds in favour of the amyloidogenic forms of TTR. It is suggested that unfolding events within the TTR monomer originate at the C-D loop of the protein, and that destabilising mutations in this region enhance the rate of TTR fibrillation. Furthermore, it is proposed that the binding of small molecule drugs to TTR stabilises non-amyloidogenic states of TTR in a manner similar to that occurring for the protective mutants of the protein.
Human transthyretin (TTR) is a 55 kDa homotetrameric protein that transports thyroxine (T₄) and retinol-binding protein (RBP) in the serum and cerebrospinal fluid (Fig. 1a–c). Wild-type (WT) TTR is intrinsically amyloidogenic, and causes amyloid fibril formation in elderly individuals, resulting in senile systemic amyloidosis (SSA)₁,₂. A large number (＞100) of TTR mutants have been identified (http://amyloidosismutations.com). The majority of these are implicated in familial amyloid polyneuropathy/amyloid cardiomyopathy (FAP/FAC) and several other forms of amyloidosis³–⁵. Of special interest are the T119M and S52P mutations. The T119M mutation is non-amyloidogenic, and heterozygous individuals carrying the T119M mutation and an amyloidogenic mutation, such as V30M, either remain asymptomatic of FAP or present a more benign form of the disease⁶,⁷. On the other hand, individuals carrying the S52P mutation develop aggressive and early-onset fatal amyloidosis⁸,⁹. Currently, only one drug—tafamidis (trade name Vyndaqel; Pfizer)—has been granted market authorisation for the treatment of TTR-FAP in adult patients having stage I symptomatic polyneuropathy¹⁰.

Considerable effort has been invested in seeking to identify the factors that underlie TTR amyloid formation including X-ray diffraction¹¹–¹³, NMR¹⁴–¹⁶, solution scattering¹⁷, and mass spectrometry (MS)¹⁸. However, a molecular level understanding of the widely different behaviours of various TTR mutants has remained elusive¹¹,¹². In this paper, native MS, neutron crystallography, and computer simulations have been used to investigate the effects of the T119M and S52P mutations on TTR stability and the molecular determinants of amyloid formation. It is found that the S52P mutation destabilises TTR by altering the stability of the CD loop in the protein monomer. In contrast, the T119M mutation stabilises the dimer–dimer interface as well as TTR’s tertiary structure. Furthermore, it is shown how the stability of TTR tetramers is coupled to those of the monomers and dimers. Finally, it is suggested that tafamidis stabilises native tetrameric TTR in a similar way to that occurring for the T119M mutant in terms of thermodynamic, kinetic and structural features. A molecular mechanism is proposed by which the vastly different behaviours of the various TTR mutants can be understood.

**Results**

**Kinetics of TTR tetramer dissociation.** Tetramer dissociation is thought to be the rate-limiting step in amyloid formation¹⁹–²². MS was used to follow the subunit exchange dynamics of TTR over several days so that the effect of the two mutations, and that of tafamidis, on the rate of dissociation of the tetramer species could be compared. These experiments relied on the use of hydrogenated (H) and deuterated (D) TTR to track otherwise identical protein chains. H- and D-TTR were mixed in an equimolar ratio and monitored over time to observe the rate of dissociation of the homo-tetramers (4H, 4D) and the rate of formation of the hetero-tetramers (2H2D, 1H3D, 3H1D; Fig. 1d and Supplementary Figure 1). The two 2H2D assemblies that can be formed may have different association constants, given that one requires only association of homo-dimers while the other requires the formation of hetero-dimers before assembly into tetramers. However, these two forms cannot be differentiated by MS, and are necessarily considered together. Since it has been shown that the kinetics of TTR subunit exchange is susceptible to isotope effects¹⁸, a deuterated TTR species ([D]S52P) was used as a reference to allow a meaningful comparison of the TTR variants. The change in relative abundance of each tetrameric species over time, and the relative association/dissociation rate constants are shown in Fig. 1e–h.

The dissociation rate of homo-tetramers in the (H)WT-(D) S52P experiment (Fig. 1e) was 0.20 ± 0.03 day⁻¹. Among the hetero-tetramer species, those containing identical dimers (2H2D; two hydrogenated and two deuterated TTR monomers) were formed at a higher rate (0.33 ± 0.04 day⁻¹) than those formed by two different dimers (3H1D and 1H3D; 0.14 ± 0.03 day⁻¹). The observed rate of dissociation of homo-tetramers in the (H)S52P-(D)S52P experiment (Fig. 1f) was about three times larger than in the (H)WT-(D)S52P (0.59 ± 0.03 day⁻¹). In this case, however, the 3H1D/1H3D species were formed faster than the 2H2D ones: 0.83 ± 0.06 day⁻¹ and 0.45 ± 0.01 day⁻¹, respectively. In the (H) T119M-(D)S52P experiment (Fig. 1g), almost no tetramer dissociation was observed over the course of eleven days—a timescale over which essentially all of the TTR would be recycled in a physiological context (the biological half-life of TTR is 1–2 days³⁵). A final experiment was carried out with (H)S52P and (D)S52P in the presence of tafamidis at a molar ratio of 1:1 (Fig. 1h). In this case, tetramer dissociation was almost eliminated by the presence of the drug. This also demonstrated that one molecule of tafamidis per tetramer was sufficient for effective stabilisation.

Overall, the MS experiments revealed that the S52P mutation increases the rate of dissociation of TTR tetramers by about a factor of three as compared with the WT (reduced tetramer stability was also observed as a function of pH—see Supplementary Figure 2a-b). In strong contrast, both the T119M mutation and tafamidis effectively abolished tetramer dissociation.

**Effect of mutations on TTR equilibria.** Free energy (G) calculations were used to study the effects of the T119M and S52P mutations on the thermodynamic stability of TTR tetramers, dimers and monomers. These calculations were based on MD simulations²⁴ and the thermodynamic cycle is shown in Fig. 2. Control over the potential energy function used allowed specific residues to be changed into other ones, so that the free energy change associated with the modifications could be calculated for the protein in its tetrameric (ΔG₄tet), dimeric (ΔG₄dime) or monomeric (ΔG₄mono) states. Differences between these ΔG values provide an estimate of the stability of the mutant tetramers, dimers and monomers relative to the WT: ΔΔG₄tet, ΔΔG₄dime, and ΔΔG₄mono respectively (Fig. 2).

The calculations suggested that the S52P mutation would not have a significant effect on the stability of the tetramers and dimers for their dissociation to dimers and monomers, respectively (ΔΔG₄tet and ΔΔG₄dime). However, a large and statistically significant ΔΔG₄mono (+2.1 kcal mol⁻¹) indicated that the introduction of the S52P mutation had a destabilising effect on the monomer with respect to the WT-TTR, corresponding to a shift towards unfolded TTR by a factor of ~34. The T119M mutation had no effect on the stability of the dimers. However, in contrast to the case for S52P, a large stabilising effect on the tetramers was observed (ΔΔG₄tet = −8.9 kcal mol⁻¹; equilibrium constant fold-change of ~3 x 10⁶). In addition, the T119M monomers were calculated to be more stable than WT monomers by 1 kcal mol⁻¹ (~5-fold increase in stability), a small but statistically significant effect.

When considering the overall process of tetramer dissociation and unfolding, the S52P mutation was estimated to shift the equilibrium toward the unfolded state by 8.1 kcal mol⁻¹ (a change of ~8 x 10⁶), whereas the T119M mutation was estimated to have the opposite effect of stabilising the tetramers by 12.4 kcal mol⁻¹ (a change of ~10⁶). Given that the two mutation sites are not in close contact, it is reasonable to assume additivity for their associated energetic effects and on this basis the data suggest that in a double S52P/T119M-TTR mutant, the T119M mutation...
A green (by superimposition of PDB-ID 6FFT), the S52 amino acid in red, and T119 in blue. The S52 amino acid in red, and T119 in blue. Respectively. Changes in relative abundance of homo- and hetero-tetrameric species over the course of 11 days are shown, along with an estimate of their association/dissociation rates (uncertainties are one standard deviation of the posterior densities)

4D (deuterated) tetramers are mixed in equal parts; following dissociation, four hybrid tetrameric species may form by the assembly of monomeric and protective effect of the T119M mutation25,26. This is in agreement with the observed in vivo mutation; this is in agreement with the observed in vivo

would be able to balance the destabilising effect of the S52P mutation; this is in agreement with the observed in vivo protective effect of the T119M mutation25,26.

In summary, the free energy calculations show that the S52P mutation results in a TTR protein having a less stable fold. However, this appears to have little direct impact on tetramer and dimer dissociation. In contrast, the T119M mutation directly affects tetramer dissociation to dimers by stabilising the tetramer species and also has the effect of stabilising the protein fold.

Neutron diffraction reveals the basis of TTR instability. In order to study the intramolecular interactions of S52P- and T119M-TTR in atomic detail, room-temperature neutron crystallography studies were carried out. The crystal structures of the S52P mutant, the T119M mutant, and the S52P mutant in complex with tafamidis, were determined at resolutions of 1.80 Å, 1.85 Å, and 2.00 Å, respectively. Most of the interaction network across the WT and mutant structures. However, crucial differences are identified as the source of the different kinetic and thermodynamic stabilities observed.

In the T119M structure, the longer Met119 side chain extends across the tyrosine-binding channel into a hydrophobic pocket surrounded by residues Leu17, Ala19, Leu110 and Val121 (Supplementary Figure 5). These interactions, as also noted in previous studies, enhance the association of two dimers and hence the overall stability of the tetramer27.

The amino acid at position 52 plays a crucial role. In the WT and the T119M mutant structures, the amide-D and the side-chain hydroxyl of the Ser50, resulting in a stable CD loop (Fig. 3a). The highly amyloidogenic S52P mutant has a proline residue at position 52 in place of the serine present in the WT. The lack of a main-chain amide-H and a side-chain hydroxyl group in the proline residue prevents the formation of two hydrogen bonds and causes a loss of stability of the protein (Fig. 3b). The distance between amide-N of residue 52 and Cα of Ser50 in the WT and T119M structures is 4.2 Å (Fig. 3a). The same distance is 0.4 Å wider in the S52P mutant, implying a looser CD loop (Fig. 3b). This destabilises the β-turn where the residue is located as well as the associated C and D strands.

In the S52P/tafamidis complex, the CD loop is not stabilised by tafamidis binding and the distance between Ser52-N and Ser50-Cα remains as 4.6 Å. In contrast to the findings of Bulawa et al.28, no water molecules were found bridging the protein and tafamidis in this complex. Instead, tafamidis induced a 180° flip to the Thr119 side chain (Fig. 3c), with the hydroxyl moiety now forming a hydrogen bond to a water molecule that bridges the two TTR dimers via Asp18; this was observed in both binding sites. Interestingly, this water molecule fills the same space that is
occupied by the Met119 side chain in the non-pathogenic T119M mutant (Fig. 3d). Grand canonical Monte Carlo calculations suggested that the stabilisation of this water molecule upon tafamidis binding may substantially contribute toward the affinity of tafamidis, by about $-2.8$ kcal mol$^{-1}$ (Supplementary Figure 4).

Collectively, the neutron structures of T119M-TTR, and S52P-TTR in apo and holo forms, have revealed that most interactions between the $\beta$-strands forming the core of the protein are conserved. However, the $\beta$-turn between the C and D strands is loosened in S52P-TTR, suggesting that this location is crucial to the reduced stability of this protein, and may provide insight to the mechanism by which the drug stabilises the TTR tetramer.

### Coupling between quaternary and tertiary structure stability

High-temperature MD simulations were used to study the effect of TTR quaternary structure on the kinetics of TTR unfolding. Simulations were carried out at 598 K for the tetramers, dimers and monomers of WT-TTR with two modern force fields (Amber99sb*-ILDNP$^{30,31}$ and Charmm36$^{32}$). As a measure of unfolding, the fraction of native contacts $Q$ was employed$^{33}$, where 1 indicates a folded protein and 0 an unfolded protein. A similar metric was used to define the fraction of monomer–monomer and dimer–dimer interface contacts retained during the high-temperature simulations (Fig. 3e; Charmm36 results in Supplementary Figure 6). At the temperature of 298 K, all monomers and interfaces were stable with $Q$ close to one for simulations of up to $1$ μs (Supplementary Figure 7).

In Fig. 3f, the degree of unfolding of TTR monomers over the course of 100 ns simulations is shown for simulations of the tetramer, dimer and monomer in solution. The monomer in solution was found to unfold faster than the monomers that were part of a dimer, which in turn unfolded faster than the monomers that were part of the tetramer. Thus, it appeared that the quaternary structure of TTR had an effect on the stabilisation of the individual monomeric chains. It is conceivable that the opposite would hold true as well: i.e., that highly stable monomeric chains (as in T119M) may enhance the stability of the tetramer, whereas unstable monomeric chains (as in S52P) are likely to have a detrimental effect on the stability of the tetramers. The same effect was observed for the stability of the monomer–monomer interface. This interface was more resistant to disruption than the dimer–dimer interface (Fig. 3e). However, when simulating the TTR dimer, the same interface became much more easily disrupted within the timeframe of the simulations (Fig. 3g).

Partial-least squares (PLS) functional mode analysis (FMA)$^{34,35}$ was used to identify regions of the TTR protein that contributed most to its unfolding while being part of a tetrameric unit. In Fig. 3h, a WT-TTR monomer is shown and colour-coded according to the root mean square fluctuations of the maximally correlated mode. The figure shows how the largest loss of native contacts observed was due to large motions in the C and D strands (Charmm36 results in Supplementary Figure 8). Similar observations were also made when calculating the fraction of native contacts by strand (Supplementary Figure 9).

These MD simulations showed that TTR tertiary structure is stabilised by the formation of tetrameric and dimeric units. Similarly, the monomer–monomer interface is stabilised by the association of TTR dimers into tetramers. Taken together, the observations explain why tetramer dissociation is the rate-limiting step in the formation of amyloid. In addition, in agreement with previous NMR data$^{16,36}$, PLS-FMA identify the C and D strands as the regions of TTR tetramers most prone to unfolding.

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**Fig. 2** Relative stability of mutant tetramers, dimers, and monomers, with respect to WT-TTR. $\Delta \Delta G_{\text{WT}}$ is the stability of the mutant tetramers with respect to WT-TTR for the process of dissociation to dimers; $\Delta \Delta G_{\text{mono}}$ represents the relative stability of the mutant dimers when they dissociate to monomers; $\Delta \Delta G_{\text{mono}}$ indicates the relative stability of the mutant monomers for unfolding; $\Delta \Delta G_{\text{unf}}$ is the relative stability of the mutant tetramers for the overall processes of tetramer dissociation and unfolding. The uncertainties shown are one standard error of the mean, obtained from ten repeated calculations.

| Tetramer          | Dimer          | Monomer         | Unfolded monomer |
|-------------------|----------------|-----------------|------------------|
| $\Delta \Delta G_{\text{WT}}$ | $2 \times \Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ | $4 \times \Delta \Delta G_{\text{WT}}$ |
| $\Delta \Delta G_{\text{MT}}$ | $2 \times \Delta \Delta G_{\text{MT}}$ | $\Delta \Delta G_{\text{MT}}$ | $4 \times \Delta \Delta G_{\text{MT}}$ |
| $\Delta \Delta G_{\text{GT}}$ | $2 \times \Delta \Delta G_{\text{GT}}$ | $\Delta \Delta G_{\text{GT}}$ | $4 \times \Delta \Delta G_{\text{GT}}$ |

| $\Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ |
|-------------------|-----------------|-----------------|-----------------|
| $\Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ |
| $\Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ |
| $\Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ | $\Delta \Delta G_{\text{WT}}$ |

**ΔΔG in kcal/mol:**

- $\Delta \Delta G_{\text{SSCP}}$ = $+0.7 \pm 0.6$
- $\Delta \Delta G_{\text{SSCP}}$ = $-0.5 \pm 0.3$
- $\Delta \Delta G_{\text{SSCP}}$ = $+2.1 \pm 0.2$
- $\Delta \Delta G_{\text{SSCP}}$ = $+8.1 \pm 0.8$

- $\Delta \Delta G_{\text{T119M}}$ = $-8.9 \pm 0.6$
- $\Delta \Delta G_{\text{T119M}}$ = $+0.2 \pm 0.2$
- $\Delta \Delta G_{\text{T119M}}$ = $-1.0 \pm 0.1$
- $\Delta \Delta G_{\text{T119M}}$ = $-12.4 \pm 0.6$
Binding of tafamidis (3MI) results in a change of orientation for residue Thr119 (cyan, before binding; green, after binding). In the S52P/tafamidis complex, the hydroxyl side chain of Thr119 forms a hydrogen bond to a water molecule.

The water molecule in the S52P/tafamidis complex occupies the same position as the side chain of Met119 residue (light orange) in the non-pathogenic T119M (not shown).

The change in the fraction of native contacts for the high-temperature simulations of tafamidis to TTR quantifies the stabilising effect of the drug on amyloid fibrillation of S52P-TTR (Supplementary Figure 2c) as well as the faster tetramer dissociation rates observed.

T119M-TTR tetramers were found to be highly resistant to dissociation. The free energy calculations estimated a stabilising effect of $8.9 \text{ kcal mol}^{-1}$ on the tetramer, which corresponds to a change in equilibrium constant of $\sim 3 \times 10^5$. This effect can be understood in terms of the increased contact area between the two dimers, which stabilises the dimer–dimer interface (Supplementary Figure 5).

In addition, the free energy calculation results suggest a tertiary fold in T119M-TTR that is more stable than in the WT by about 1 kcal mol$^{-1}$. As illustrated in Fig. 4b, tetramer stabilisation directly reduces the population of amyloidogenic building blocks. Furthermore, a stable tertiary structure can protect against the unfolding of monomeric TTR. Finally, the coupling between TTR quaternary structure and the stability of the TTR fold suggested by the simulations indicates that the highly stable T119M-TTR tetramer reduces the probability of the CD strands unfolding (Fig. 4b).

Stabilisation of the native tetrameric structure by small molecule binding to the T4 binding sites of TTR is a well-cited rationale for the inhibition of TTR amyloidogenesis. MS kinetic experiments have shown how tafamidis, like the T119M mutation, strongly inhibits tetramer dissociation. The binding affinity of tafamidis to TTR quantifies the stabilising effect of the drug on TTR tetramers, and was measured to be $\sim 12 \text{ kcal mol}^{-1}$ ($K_d = 2$).
The stabilising effect of T119M on the tetramer (with respect to the dimer) was found to be about 9 kcal/mol. However, considering the further gains in TTR fold stability conferred by the mutation ($\Delta \Delta G_{T119M}$ (mono)), the overall thermodynamic effect is very similar to that of the tafamidis-S52P complex, with $\Delta \Delta G_{T119M}$ (unf) $\approx -12$ kcal mol$^{-1}$. Tafamidis therefore stabilises native TTR thermodynamically and kinetically in a manner that is comparable to T119M. However, it is difficult to predict the extent to which the two separate effects of tetramer and fold stabilisation may impact on the in vivo protection against amyloidogenesis. Structurally, the binding of tafamidis results in the reorientation of Thr119 and the emergence of a water molecule in the binding pocket. This water molecule is located in the same position as the side chain of Met119 in the T119M mutant.

Fig. 4 Proposed model of mutational effect on TTR stability. Shorter arrows indicate a lower propensity to association/folding. Red arrows reflect the qualitative change in reaction rates for the mutants with respect to WT-TTR. a) The S52P mutation increases the likelihood of partial and full unfolding of TTR monomeric units. This, in turn, also leads to a lower stability of TTR multimeric assemblies; hence partially unfolded dimers and monomers, and fully unfolded monomers, are generated at a higher rate. Some or all of these species are then removed from solution via the formation of amyloid fibrils. b) In contrast to S52P, the T119M mutation stabilises the folded monomeric forms of TTR. Most importantly, this mutation has a large stabilising effect on TTR tetramers, which are believed not to form fibrils. Furthermore, it is proposed that the higher stability of the tetramer also has the effect of further stabilising the monomer fold
level detail of direct relevance to TTR amyloidogenicity, and have provided a framework for further investigation into the effects of residue mutation on TTR’s states and their equilibria, and for the development of novel targeted therapies for FAP and SSA.

Methods

Protein preparation and crystallization. The cDNA corresponding to the gene coding for the 127 amino acids of human transthyretin (TTR) protein was cloned into a pET-M11 vector encoding an N-terminal His tag and a TEV cleavage site (EMBL Protein and Purification Facility, Germany) and then expressed in E.coli BL21 (DE3) cells (Invitrogen). The SS2P and T119M mutants were generated using QuickChange Lightning Multi Site-Directed Mutagenesis kit (Strategene). WT-TTR cDNA sequence and the sequences of primers used for SS2P and T119M mutations are shown in Supplementary Table 1. Deuterated protein was produced in the Deuteration Laboratory (D-Lab) platform within ILL’s Life Sciences Group48. Cells were adapted to deuterated E. coli minimal medium and grown in a fed-batch fermenter culture at 30 °C using d3-glycerol (99% deuterium; Euriso-top) as the only carbon source. H-TTRs and D-TTRs were purified in an identical manner. Cell paste was resuspended homogeneously in lysis buffer (20 mM Tris pH 8, 250 mM NaCl, 3 mM imidazole) in the presence of EDTA-free protease inhibitor cocktail (Roche). The cells were lysed by ultrasonication on ice at 50% amplitude and 25 sec pulses. The lysate was cleared by centrifugation at 18,000 rpm for 4 °C for 30 min. The supernatant containing crude protein extracts was recovered and purified using benchtop gel-permeation chromatography. Nickel-nitrilotriacetic acid (Ni2+–NTA) resin was pre-washed with lysis buffer and left in incubation with the proteins for at least an hour to increase binding efficiency. The protein resin mix was then loaded into 10 ml disposable column. The column was washed three times with wash buffer (20 mM Tris pH 8, 15 mM imidazole, NaCl at 500 mM, 1 M, and 250 mM, respectively) before the protein was eluted (elution buffer: 20 mM Tris pH 8, 250 mM NaCl, 250 mM imidazole). Fractions containing protein were then pooled. Further TTR protein treatment included removal His–tagged protein into gel filtration buffer (10 mM Tris pH 7.5, 50 mM NaCl, 1 mM DTT) overnight at 4 °C. Protein was separated from the cleared poly-histidine tail using the same gravity-flow column described above. Flowthrough from the column was concentrated, filtered (with 0.22 μm membrane pore size) and loaded onto Superdex 75 HiLoad 16/600 gel filtration column (GE Healthcare) running at 1 ml/min at room temperature. Peak fractions were pooled and concentrated using Amicon Ultra centrifugal filter units (Millipore) and stored at −20 °C. Analysis was carried out on 12% Tris-Tricine gel after each step of purification to monitor the purity of the protein. The details of the expression and purification have been described previously18,49. While the deuterated TTR was expressed under conditions where only carbon source was the one used at BIODIFF was used. Data were recorded at room temperature on capillary-mounted crystals. Data were processed to the same maximum resolution of the corresponding neutron data with XD58, scaled and merged with SCALA53, and converted to structure factors using TRUNCATE in the CCP4 suite59. Relevant data collection statistics are summarised in Supplementary Table 2.

X-ray data collection and processing. X-ray diffraction data of the crystals of all the three different MWs of TTR in the presence of beta-lactamase was collected with the use of favorite reflection at 100 K at the European Synchrotron Research Facility (ESRF). Grown using a heavily attenuated X-ray beam (1.96% for SS2P, 19.17% for T119M; 9.83% for SS2P/tafamidis) of wavelength 0.9763 Å. For SS2P and SS2P/tafamidis complex, the same crystals used for data collection at LADI-III was used; whereas for T119M, a crystal from the same crystallization drop as the one used at BIODIFF was used. Data were recorded at room temperature on capillary-mounted crystals. Data were processed to the same maximum resolution of the corresponding neutron data with XD58, scaled and merged with SCALA53, and converted to structure factors using TRUNCATE in the CCP4 suite59. Relevant data collection statistics are summarised in Supplementary Table 2.

Joint neutron and X-ray structure refinement. The PDB structures SCLX (resolved X-ray models of TTR crystals collected at 100 K) was used as starting models for joint X-ray and neutron refinement against the datasets of SS2P and SS2P/tafamidis; and 5CM1 (resolved X-ray models of TTR S119M; data collection at 100 K) was used for the datasets of T119M. The phenix.refine program59 in the PHENIX package59 was used for refinement. The preparation of the starting model, the model building and the modelling of solvent were performed as detailed in Haupt et al.54. Using the ReadySet option in PHENIX59, exchangeable hydrogen and deuterium atoms were placed at appropriate sites of the protein models with deuteriums elsewhere. For positions where both hydrogen and deuterium atoms were modelled, the occupancies of both were set to 0.5 and then those occupancies were refined, with their total occupancy constrained to 1. D-O2 molecules were added using ReadySet based on the positive neutron scattering length density in F–F–maps. Coor54 was used for model modifications, such as addition of solvent molecules, and rotamer- and torsion-angle adjustments, according to positive and negative nuclear scattering length density in both 2F–F– and F–F– maps. The final refinement statistics are summarised in Supplementary Table 3.

Monitoring subunit composition of tetrameric TTR by native MS. The details of the instrumental arrangements and sample preparation protocols have been described previously37. MS analyses were carried out on a quadrupole time-of-flight mass spectrometer (Q-TOF Ultima, Waters Corporation, Manchester, U.K.) that was modified for the detection of high mass~58. Coeluting peptides were resolved using benchtop gravity-centrifugal filtration buffer (10 mM Tris pH 7.5, 50 mM NaCl, 1 mM DTT) overnight at 4 °C. Protein was separated from the cleared poly-histidine tail using the same gravity-flow column described above. Flowthrough from the column was concentrated, filtered (with 0.22 μm membrane pore size) and loaded onto Superdex 75 HiLoad 16/600 gel filtration column (GE Healthcare) running at 1 ml/min at room temperature. Peak fractions were pooled and concentrated using Amicon Ultra centrifugal filter units (Millipore) and stored at −20 °C. Analysis was carried out on 12% Tris-Tricine gel after each step of purification to monitor the purity of the protein. The details of the expression and purification have been described previously37. While the deuterated TTR was expressed under conditions where only deuterium atoms were present, protonated (H-based) solutions were used during purification. Prior to protein crystallization, the purified protein was buffer-exchanged using a deuterated solution so that the labile proton atoms acquired during the purification steps that involved hydrogenated solutions were replaced by deuterium atoms. Therefore, for the deuterium detection, the protein solution was dissolved in 100% deuterated dimethyl sulfoxide (D-DMDSO) for crystallization. All crystals were grown by sitting-drop vapour diffusion at 18 °C using deuterated protein and deuterated solution. The TTR SS2P mutant crystal (~0.60 mm2) were grown in 1.9 M malonate pD 6.4 with a protein concentration of 25 mg/ml (drop volume 50 μl; protein-to-buffer ratio of 7:5), while the T119M mutant crystal (~0.80 mm2) were in 1.9 M malonate pD 5.9 with 40 mg/ml protein concentration (drop volume 40 μl; protein-to-buffer ratio of 1:1). For neutron data collection, the crystals were mounted in quartz capillaries and surrounded by a small amount of mother liquor from the crystallization well. The capillaries were sealed tightly using wax to eliminate the diffusion of gas and atmospheric water. Neutron data collection and processing. Details of the neutron data collection for WT-TTR have been reported by Haupt et al.48. Neutron quasi-Laue diffraction data from the crystals of the SS2P mutant and the SS2P/tafamidis complex were collected at room temperature using the LADI-III diffractometer49 at the Institute Laue-Langevin (ILL), Grenoble. For the SS2P mutant, a neutron wavelength range (Δλ/λ = ±30%) of 2.9–3.9 Å was used, with data extending to 1.8 Å resolution. As is typical for a Laue experiment, the crystal was held stationary at different ϕ (vertical rotation axis) settings for each exposure. A total of 26 images (exposure time of 18 h per image) were collected from different crystal orientations for the SS2P/tafamidis complex, a neutron wavelength range of 2.7–3.6 Å was used with data extending to 2.0 Å resolution. In total, 13 images were collected (exposure time of 8 h per image) from three different crystal orientations. The neutron diffraction images were indexed and integrated using the LAUE suite program LAUEGEN51. The program SCALE52 was used to determine the wavelength-normalisation curve using the intensity of symmetry-equivalent reflections measured at different wavelengths and normalised for associated wavelength-normalisation prior to c=y0 and y>c=y0 were used for dissociation, and between yc=y0 and 100% for association. For k, a half normal prior with mean of 0 min–1 and standard deviation of 10 min–1 was used. White

Neutron monochromatic diffraction data for the T119M mutant crystal were collected at room temperature using the instrument BIODIFF44 operated by the Forschungsrecht Zirkel München II (FRM II) and the Jülich Centre for Neutron Science (JCSN) at Garching. For data collection, the wavelength was set at 2.67 Å (Δλ/λ = 2.9%). A total of 202 frames were recorded with a rotation range of 0.35° and an exposure time of 53 min per frame. The diffraction data were indexed and integrated using HKL200055 and scaled using program18,49. The two files of HKL2000 in MTZ format by using scalepack2mtz program in the CCP4 suite59. Relevant data collection statistics are summarised in Supplementary Table 2.
noise was modelled with a half normal prior with zero mean and standard deviation of 10%. The posterior estimate of the parameters was obtained by drawing 20,000 Markov chains from Multivariate Monte Carlo (MCMC) using the No-U-Turn Sampler (NUTS) algorithm. Five thousand tuning steps were carried out and discarded before collecting the posterior samples used for the estimate. The maximum a posteriori estimate (MAP) of the parameter space was passed to the sampler as the starting value. The mean and standard deviation of the rate constants (k) from their posterior distributions are reported.

High-temperature molecular dynamics simulations. Molecular dynamics (MD) simulations were performed with Gromacs 2016. The starting structure for the simulations was the WT (PDB-ID 4PVM) variant of TTR protonated at pH 7 with pdb2gmx. The dimeric and monomeric models were obtained from the same neutron crystal structure—dimer for the dimers, and A and B were used for the monomers—chain A was used. The protein was modelled with the Amber99SB*-ILDNP topology and the Charmm36 (Nov. 2016) force field, and water molecules with the TIP3P model. The protein was solvated in a dodecahedral box with periodic boundary conditions and a minimum distance between the solute and the box of 12 Å. Sodium and chloride ions were added to neutralise the systems at the concentration of 0.15 M.

Energy minimisation was performed using a steepest descent algorithm with force tolerance of 10 kJ mol⁻¹ nm⁻¹ for a maximum of 10,000 steps. 1 ns NVT and then NPT equilibrating simulations were performed with all solute heavy atoms restrained with a force constant of 20 kJ mol⁻¹ nm⁻². In these simulations, the temperature was coupled with the stochastic v-rescale thermostat by Russi et al. at the target temperature of 298 K; the pressure was controlled with the Berendsen weak coupling algorithm at a target pressure of 1 bar. The particle mesh Ewald (PME) algorithm was used for electrostatic interactions with a real space cut-off of 11 Å, a spline order of 4, a relative tolerance of 10⁻⁶ and a Fourier grid spacing of 1.2 Å. The Verlet cut-off scheme was used for non-bonded interactions cut-off of 11 Å and a buffer tolerance of 0.005 kJ mol⁻¹ nm⁻². Bonds involving hydrogens were constrained with the LINCS algorithm. Ten equilibrium simulations of 10 ns duration were then initiated from the last frame of the short (0.5 ns) equilibration simulations. From each of the ten equilibration simulations, a non-equilibrium trajectory was spawned every 0.2 ns, for a total 50 trajectories per equilibrium simulation and 500 trajectories overall. Forward and reverse transformations were performed for both mutants, with 500 non-equilibrium trajectories per transformation: WT→S52P, S52P→WT, WT→T119M, T119M→WT. Coordinates for hybrid residues were built with pmx57 after extracting the starting configurations of the systems. Energy minimisation was performed on the dummy atoms only, before equilibrating velocities with a 10 ps simulation. Then, the non-equilibrium alchemical transformation was performed over 100 ps. The non-equilibrium simulations were performed using a custom version of Gromacs 4.6 that implements the soft-core potential described in Gapsys et al., where both vdW and Coulombic interactions were soft-cored. Free energy differences were estimated using the Bennet's Acceptance Ratio69, and implemented in pmx75. Uncertainties in the ΔG values were calculated by taking the standard error of the BAR estimate from the ten independent equilibrium simulations and related non-equilibrium trajectories.

Grand canonical monte carlo calculations. The simulation package ProtoMS 3.3 was used for Grand Canonical Monte Carlo (GCMC) calculations and data analysis. The starting tetramer structures for the calculations were the WT-TTR (PDB-ID 4PVM), S52P-TTR (PDB-ID 5NFW), S52P-TTR/tafamidis (PDB-ID 6FFT) and T119M-TTR (PDB-ID 5NFE) with protonations as resolved experimentally. Proteins were modelled with the Amber ff14SB force field, water with the TIP3P model, and tafamidis with the GAFF/AM1-BCC force field.80,81 For the calculation of the stability of W17, a GC box was created around the water molecule by adding 2 Å padding in all three dimensions and used for both the calculations in S52P-TTR and S52P-TTR/tafamidis. For the calculation of the monomer–monomer interface hydration free energy, the grand canonical box was defined so to encompass the interface containing the conserved water molecules of interest; the same box was used for all proteins, which had been previously aligned. Because the hydration free energy results might be sensitive to the protonation state of the histidine residues in proximity of these conserved water molecules, from each monomeric structure we removed the other two monomers and the three amino acids at positions 52 and 119. Thus, for each TTR variant, we performed three sets of calculations, based on each neutron structure and its respective protonation states of His51, His56, His88 and His90.

Protein residues that were further than between 16 and 20 Å away from the grand canonical region were removed, with the exact distance chosen to retain whole residues. The systems were then solvated up to a radius of 30 Å around the grand canonical region. All simulations were carried out at 298 K, and a 10 Å cut off was applied to the non-bonded interactions. Before initiating the GCMC simulations, the systems were equilibrated using 50 million (M) solvent-only moves in the canonical ensemble, so to equilibrate the water around the proteins. Water molecules present in the pre-defined GCMC box were then removed; this set of coordinates represented the starting point of the GCMC simulations.

For the calculation of the monomer–monomer interface hydration calculation, a set of 32 simulations were performed at a range of Adams values from −29 to +2 at unit increments, so the calculation of the stability of W17 using a forcing between 18 and 0 were used instead. For each window, 15 M equilibration moves were performed only on the grand canonical solute, with insertion, deletion and translation/rotation moves generated at the same ratio. An additional 5 M equilibration moves followed where the protein and solvent molecules were also sampled, before starting the production simulation of 50 M moves. Half of the MC moves were dedicated to the grand canonical water molecules, and the other half was split between protein residues and solvent in proportion to the number of solvent molecules and protein residues, according to the ratio of 1.5: Hamiltonian exchange was employed, with exchanges being performed every 0.2 M steps. Data for all simulations, number of GC solute moves and number of C138 moves were saved to file every 0.1 M moves.

The above procedure was repeated three times for the monomer–monomer interface hydration calculation (i.e. 9 calculations for each TTR variant, three repeated calculations per starting structure) and ten times for the W17 stability calculation. All simulations were performed with the AMBER76 software, and the binding free energy of individual water molecules, were computed using grand canonical integration (GCI) as described in Ross et al.22. GCI was performed using

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the calc_gei.py script that is part of the ProtomS 3.3 tools, with the data from all repeats being analysed together. The amount of data discarded as equilibration was determined using the equilibration detection tool available in calc_series.py. The same amount of data was discarded from all windows after determining the average number of moves needed for equilibration.

Data availability
Atomic coordinates and diffraction data have been deposited in the Protein Data Bank (accession codes 5NFW for S52P-TTR, 5NFE for T119M-TTR, and 4FTF for S52P-TTR/tafamidis complex). Other data are available from the corresponding author upon reasonable request.

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