The molecular interaction of 4′-iodo-4′-deoxydoxorubicin with Leu-55Pro transthyretin 'amyloid-like' oligomer leading to disaggregation

Maria Paula SEBASTIÃO*, Giampaolo MERLINI†, Maria João SARAIVA‡§ and Ana Margarida DAMAS*§[1]

*Molecular Structure Unit, Instituto de Biologia Molecular e Celular, Rua do Campo Alegre, no. 823, 4150 Porto, Portugal, †Biotechnology Research Laboratories, University of Pavia, 27100 Pavia, Italy, ‡Amyloid Unit, Instituto de Biologia Molecular e Celular, Rua do Campo Alegre, no. 823, 4150 Porto, Portugal, and §Instituto de Ciências Biomédicas de Abel Salazar, University of Porto, 4150 Porto, Portugal

The crystal structure of the amyloidogenic Leu-55Pro transthyretin (TTR) variant has revealed an oligomer structure that may represent a putative amyloid protofibril [Sebastião, Saraiva and Damas (1998) J. Biol. Chem. **273**, 24715–24722]. Here we report biochemical evidence that corroborates the isolation of an intermediate structure, an 'amyloid-like' oligomer, which is most probably present in the biochemical pathway that leads to amyloid deposition and that was isolated by the crystallization of the Leu-55Pro TTR variant. 4′-Iodo-4′-deoxydoxorubicin (IDOX) is a compound that interacts with amyloid fibrils of various compositions and it has been reported to reduce the amyloid load in immunoglobulin light chain amyloidosis [Merlini, Ascari, Amboldi, Bellotti, Arbustini, Perfetti, Ferrari, Zorzoli, Marinone, Garini et al. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 2959–2963]. In this work, we observed that the monoclinic Leu-55Pro TTR crystals, soaked with IDOX, undergo rapid dissociation. Moreover, under the same conditions, the orthorhombic wild-type TTR crystals are quite stable. This is explained by the different TTR conformations isolated upon crystallization of the two proteins; while the Leu-55Pro TTR exhibits the necessary conformation for IDOX binding, the same structure is not present in the crystallized wild-type protein. A theoretical model concerning the interaction of Leu-55Pro TTR with IDOX, which is consistent with the dissociation of the amyloid-like oligomer, is presented. In this model the IDOX iodine atom is buried in a pocket located between the two β-sheets of the Leu-55Pro TTR monomer with the IDOX aromatic-moiety long axis nearly perpendicular to the direction of the β-sheets.

Key words: amyloid inhibitor, crystallography, molecular modelling, transthyretin–anthracycline complex.

INTRODUCTION

Transthyretin (TTR) amyloid fibril formation is an *in vivo* polymerization process, whereby a normally soluble human plasma protein, TTR, is converted into an insoluble cross-β fibrillar quaternary structure. The biochemical process that converts the plasma TTR into amyloid fibrils is complex and probably involves several quaternary structural intermediates. Therefore, the isolation and structural characterization of those intermediates is of crucial importance in order to arrest the pathological process at an early stage of the disease.

TTR, also known as thyroxine-binding pre-albumin, is a transport plasma protein present in mammals, birds and reptiles. The protein binds the complex retinol–retinol binding protein, thyroxine and related halogenated compounds [1,2]. It has a tetrameric structure with an extended β-sheet conformation as revealed by X-ray crystallography [3]. Each monomer is composed of two four-stranded β-sheets (strands DAGH and CBEF) which in the dimer extend to two eight-stranded β-sheets (strands DAGHH′G′A′D′ and CBEFF′E′B′C′, where ′ indicates a different monomer). H-bonds between loop AB and strand H from two symmetrically related dimers lead to the tetrameric protein.

Several point mutations have been identified in the protein, most of them associated with familial amyloidotic polyneuropathy and/or cardiomiopathy [4]. One of them, Leu-55Pro TTR, is currently receiving much attention, because it causes a highly aggressive form of familial amyloidotic polyneuropathy [5] and it is the only human mutant identified so far that crystallizes non-isomorphously with wild-type TTR [6].

The crystallization and X-ray-crystal-structure determination of the Leu-55Pro TTR variant have revealed a major structural change, the disruption of the monomer D strand. This change results in different crystal intermolecular contacts, which are responsible for the assembly of an oligomer structure that may represent a putative intermediate structure in the biochemical pathway that leads to amyloidosis. Therefore, it is possible that the Leu-55Pro TTR variant is already in an 'amyloid-like' conformation under the conditions *in vitro* that are generally used to keep the wild-type protein in its native state and that this conformation was isolated by the crystallization process. Furthermore, the Leu-55Pro TTR crystals were obtained from a protein preparation that was Thioflavin-T-positive. This thiazole dye binds amyloid fibrils but it does not bind soluble wild-type TTR. Hence, it is used as an amyloid marker [7].

In order to further characterize the isolated Leu-55Pro TTR oligomer, we performed comparative ligand-binding studies with an anthracycline compound, 4′-iodo-4′-deoxydoxorubicin (IDOX), which has been used in patients with monoclonal immunoglobulin light chain (AL) amyloidosis. Recently, it was reported that IDOX binds TTR amyloid deposits and disrupts the fibrillar structure, but not the soluble TTR protein [8]. In fact, IDOX binds strongly to five types of naturally occurring amyloid fibril: AL, amyloid A, TTR (Val30Met variant), β-protein (Alzheimer's disease) and β2-microglobulin. It has been hypothesized that IDOX increases the solubility of amyloid deposits and it provides a therapeutic strategy for intervention in amyloidosis [9,10]. However, the interatomic interactions of IDOX with TTR amyloid are still not established. In this study,

Abbreviations used: IDOX, 4′-iodo-4′-deoxydoxorubicin; TTR, transthyretin; AL, immunoglobulin light-chain; GRAMM, Global Range Molecular Matching.

[1] To whom correspondence should be addressed (e-mail amdamas@ncc.up.pt).

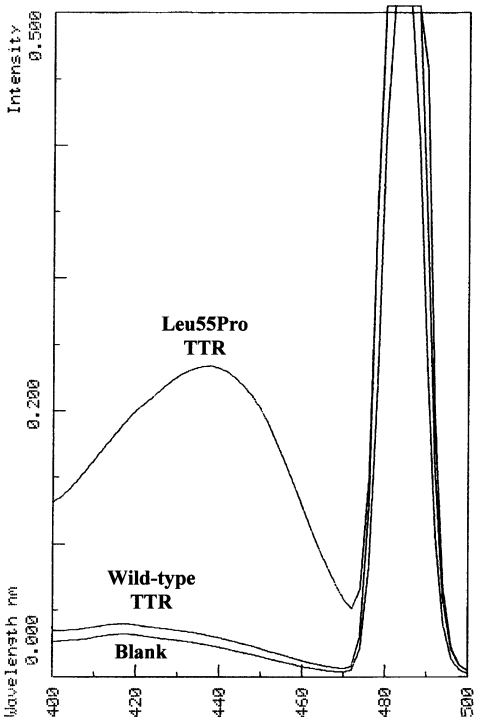

Figure 1 Thioflavine-T-binding assay for the wild-type and Leu-55Pro TTR preparations used for crystallization

we discuss the interaction between IDOX and the Leu-55Pro TTR oligomer, isolated by crystallization.

MATERIALS AND METHODS

Materials and software

IDOX was provided kindly by Pharmacia and Upjohn (Nerviano, Italy). The crystallization chemicals were from Hampton Research (Laguna Niguel, CA, U.S.A.). The crystallization plates were from Charles Supper Company (Natick, MA, U.S.A.).

The molecular-modelling programs GRAMM (Global Range Molecular Matching) and LIGIN were kindly made available to us by Dr I. A. Vakser (Medical University of South Carolina, Charleston, SC, U.S.A.) and Dr V. Sobolev (Weizmann Institute of Science, Rehovot, Israel). The graphics software program XtalView was from Dr D. E. McRee (The Scripps Research Institute, La Jolla, CA, U.S.A.).

Purification and crystallization of wild-type and Leu-55Pro TTR

Wild-type and Leu-55Pro TTR isolation was performed as described previously [11]. Briefly, the recombinant proteins were obtained by osmotic shock of *Escherichia coli* cells, purified by ion-exchange chromatography on a DEAE-cellulose column and preparative gel electrophoresis in a native Prosieve agarose gel, followed by electroelution. The TTR fraction was re-chromatographed by gel filtration in a 50 cm × 1.5 cm Bio-Rad column packed with Biogel P-100 (Bio-Rad, Hercules, CA, U.S.A.). The purity of the protein samples was confirmed by SDS/PAGE (12 % gel). Protein concentration was determined using bicinchoninic acid (BCA assay reagent, Pierce) with BSA as standard. The isolated proteins were stored at −20 °C in double-distilled deionized water, pH 5.5.

Wild-type and variant TTR preparations were tested for Thioflavine-T binding before crystallization, with the quantitative Thioflavine-T fluorimetric assay, established by Bonifácio et al. [12]. Fluorescence spectra were recorded on a Jasco FP-770 spectrofluorometer at 25 °C with an assay volume of 1 ml. The excitation spectra (400–500 nm) were taken with emission collected at 482 nm. Excitation and emission slits were set at 5 and 10 nm, respectively. The reaction mixture contained 30 μM Thioflavine T and 50 mM glycine/NaOH buffer, pH 9.

Wild-type TTR crystals were obtained by vapour diffusion in sitting drops with equal amounts of 15 mg/ml protein solution and 200 mM citrate buffer/47 % ammonium sulphate/12 % glycerol, pH 5.3, at room temperature. Leu-55Pro TTR crystals were grown at 14 °C in sitting drops containing a 1:1 mixture of 12 mg/ml protein in water and the reservoir solution {100 mM Hepes/45 % ammonium sulphate/7 % poly(ethylene glycol) 400, pH 7.5 [6]}.

IDOX binding to Leu-55Pro and wild-type crystals

The IDOX stock solution was prepared with 0.9 % NaCl to a final concentration of 496 μM, as determined by spectrophotometry at 478 nm, using an absorption coefficient of 1.33×10^4 $M^{-1} \cdot cm^{-1}$. Leu-55Pro TTR and wild-type crystals were soaked in their mother liquor containing 150 μM IDOX (molar ratio of IDOX/TTR, 10^3:1). The observed behaviour was recorded photographically using a DP10 Olympus camera and a BX50 Olympus microscope.

Wild-type TTR–IDOX complex screening by X-ray diffraction

Wild-type crystals were soaked with 150 μM IDOX in their mother liquor for 24 h. Diffraction data were collected from one of these crystals at 4 °C using the synchrotron beamline X11 equipped with an imaging-plate scanner, at the EMBL Hamburg outstation (Hamburg, Germany). The determination of the crystal orientation and integration of the reflections were performed with the DENZO and SCALEPACK programs [13].

Refinement was carried out with X-PLOR [14] using the wild-type TTR co-ordinates deposited in the Protein Data Bank under entry code 1tta [15]. The initial model contained only the protein atoms (water molecules were removed) and after one X-PLOR cycle the crystallographic *R*-factor dropped from 38.7 to 22.7 % for all data from 10- to 1.8-Å resolution.

The difference Fourier map with coefficients $(F_{obs} - F_{calc})$ and ϕ_{calc} (where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively, and ϕ_{calc} is the calculated structure factor phase angle) was then calculated and analysed in order to find the putative IDOX binding site.

Molecular modelling

A three-dimensional structural model of the Leu-55Pro TTR–IDOX complex was constructed by ligand docking using the surface complementarity algorithm implemented in the GRAMM program [16]. This program only requires the atomic co-ordinates of the receptor and ligand and does not need any prior knowledge of the binding site. GRAMM performs a search for the maximum geometric surface overlap between the receptor and the ligand, through the relative translations and rotations of the molecules. It explicitly introduces long-distance atom–atom potentials for ligand–receptor interactions, which result in the averaging of the energy potential at a given point. The intermolecular energy calculation with this long-distance force field delivers the global minima, which has been shown to correspond

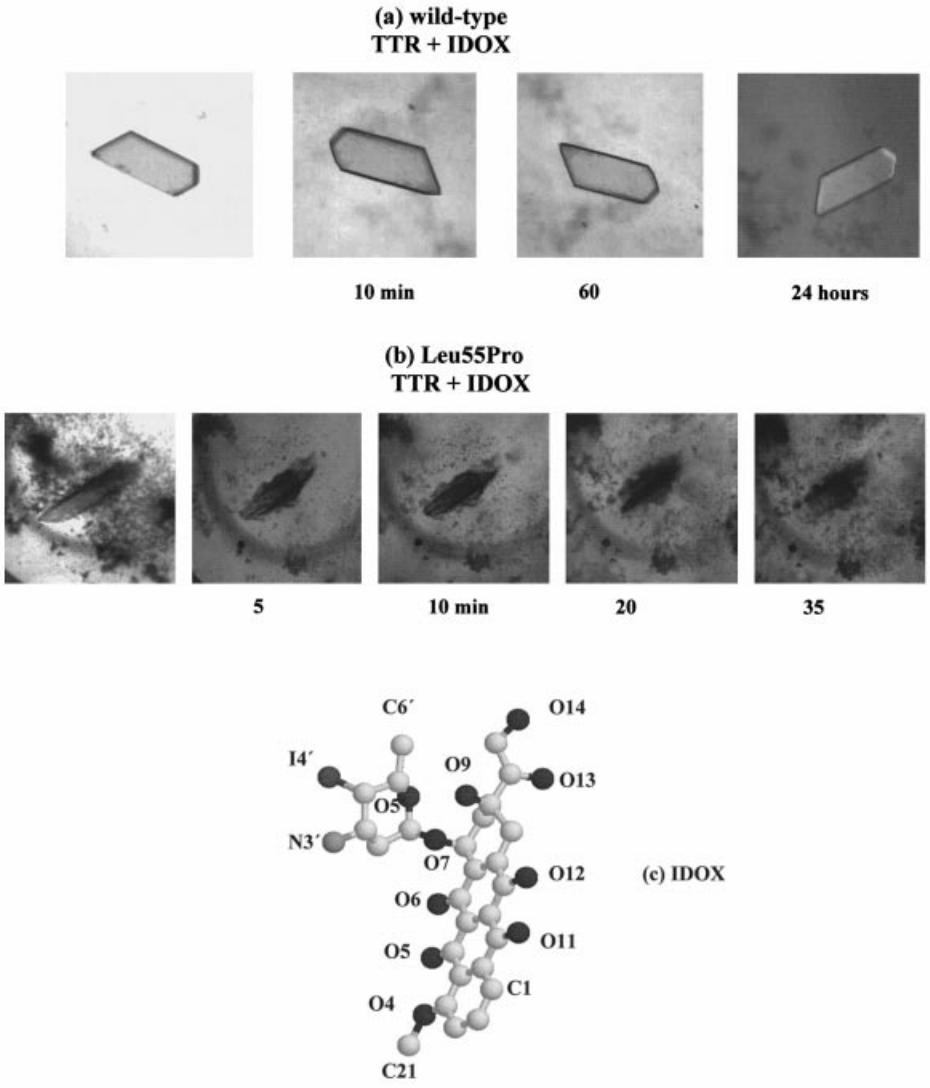

Figure 2 Wild-type and Leu-55Pro TTR crystal interaction with IDOX

(**a**) A wild-type TTR crystal, space group $P2_12_12$, with cell dimensions a = 43.87 Å, b = 85.72 Å and c = 66.22 Å, was soaked with IDOX and the observed interaction was recorded photographically, as described in the Materials and methods section. (**b**) Leu-55Pro TTR crystal, space group C2, with cell dimensions a = 149.99 Å, b = 78.74 Å, c = 98.95 Å and $\beta = 100.5$ °. (**c**) Molecular formula of IDOX.

to the experimentally determined positions of the ligand and in a number of tests suppresses local minima, or false positive fits.

Docking was performed with GRAMM, at high resolution, and with the atomic co-ordinates of Leu-55Pro TTR and IDOX deposited at the Protein Data Bank under entry codes 5ttr and 1imr [17], respectively. We only used the atomic positions of the protein atoms of Leu-55Pro TTR and the IDOX atomic co-ordinates (the oligonucleotide co-ordinates were removed from the Protein Data Bank entry 1imr). The docking parameters were: step of the grid, 1.7 Å; repulsion part of the potential, 30; and interval for rotations, 10 °. The 100 lowest energy matches were analysed with the graphics software package XtalView [18]. The first solution, corresponding to the lowest energy minimum, was considered and further refined by complementarity optimization using LIGIN. The docking algorithm implemented in LIGIN has been described in detail elsewhere [19]. Briefly, a large number of ligand positions is generated at the putative binding site of the receptor. Each of these starting positions is then subjected to a complementarity-optimization procedure and further refinement by optimization of the hydrogen bonds formed. The best solution from LIGIN, obtained after geometric and chemical complementarity between the ligand (IDOX) and the receptor (Leu-55Pro TTR), corresponds closely to the lowest energy minimum found by GRAMM.

The co-ordinates from the Leu-55Pro TTR–IDOX complex have been deposited in the Protein Data Bank at Research Collaboratory for Structural Bioinformatics (RCSB) under entry code 1F64.

RESULTS AND DISCUSSION

IDOX binding to Leu-55Pro and wild-type TTR crystals

The halogenated anthracycline derivative IDOX binds several types of amyloid fibril and produces significant clinical benefits

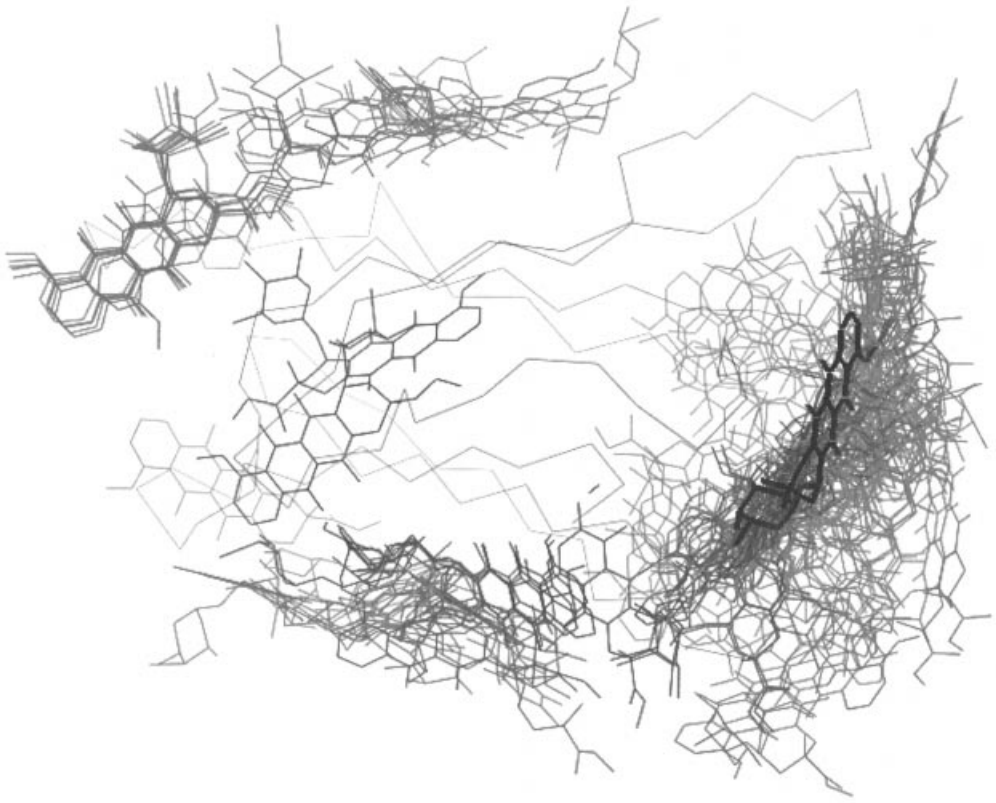

Figure 3 Leu-55Pro TTR–IDOX models obtained with GRAMM

The Leu-55Pro TTR structure is represented as a Cα trace and the IDOX clusters are also illustrated. The major cluster occurs around the lowest-energy Leu-55Pro TTR–IDOX match, which is represented in bold.

in patients with AL amyloidosis [10]. Furthermore, recent studies on TTR amyloid fibrils indicate that IDOX strongly interacts with TTR amyloid and disrupts the fibrillar structure into amorphous material [8]. These observations prompted us to investigate in detail the interaction between IDOX and the crystalline Leu-55Pro TTR 'amyloid-like' conformation.

As reported previously [20] the Leu-55Pro TTR variant is the only naturally occurring human TTR mutant that crystallizes non-isomorphously with wild-type TTR at physiological pH. Furthermore, the protein preparations used for crystallization were tested with Thioflavin T and it was observed that the Leu-55Pro TTR preparation binds the dye, whereas the wild-type preparation is Thioflavin-T-negative (Figure 1). This clearly indicates the presence of an amyloid-like structure in the Leu-55Pro TTR preparation, which was confirmed by our previous reported X-ray-crystal-structure analysis of this variant and by a comparison of amyloidogenicity for different TTR variants [12].

This fact, together with other unique biochemical and structural properties which characterize the Leu-55Pro TTR mutant [21], led us to further investigate IDOX binding to this variant. The interaction of wild-type TTR crystals with IDOX was also studied in order to compare the putative binding of this halogenated anthracycline derivative. Although the concentration of IDOX reached in plasma of AL patients is in the 7–10 μM range, a higher amyloid local concentration is expected due to the high affinity of the compound for the amyloid fibril structure, demonstrated both *in vitro* and *in vivo* [10]. In the crystallographic studies we used an excess of IDOX and attempts to study the complex between IDOX and TTR were made using crystals that were soaked in their mother liquor containing 150 μM IDOX. It was observed that the Leu-55Pro TTR crystals started immediately to dissolve and after 35 min there was only a precipitate. On the contrary, the wild-type TTR crystals remained stable under the same experimental conditions (Figure 2a).

One wild-type TTR crystal soaked with 150 μM IDOX for 24 h (Figure 2a) was mounted in a glass capillary and X-ray-diffraction data were collected using synchrotron radiation. A 90 % complete data set of up to 1.8-Å resolution was obtained, with an R_{merge} value of 7.6 %. Crystallographic refinement and difference Fourier analysis, performed as described in the Materials and methods section, indicate that IDOX does not bind wild-type TTR crystals, because no detectable electron density corresponding to IDOX was found.

However, when soaked with IDOX at the same concentration, the Leu-55Pro TTR crystals dissociate rapidly, as illustrated in Figure 2(b). Thus IDOX seems to react strongly with the crystallized Leu-55Pro TTR variant and not with the wild-type protein. In order to reveal the putative Leu-55Pro TTR residues that interact with IDOX, molecular-modelling studies were performed, as described in the Materials and methods section.

Model of the Leu-55Pro TTR–IDOX complex

Based on the previously reported Leu-55Pro TTR and IDOX crystal structures, we have performed a theoretical calculation of possible binding sites using the molecular-modelling program

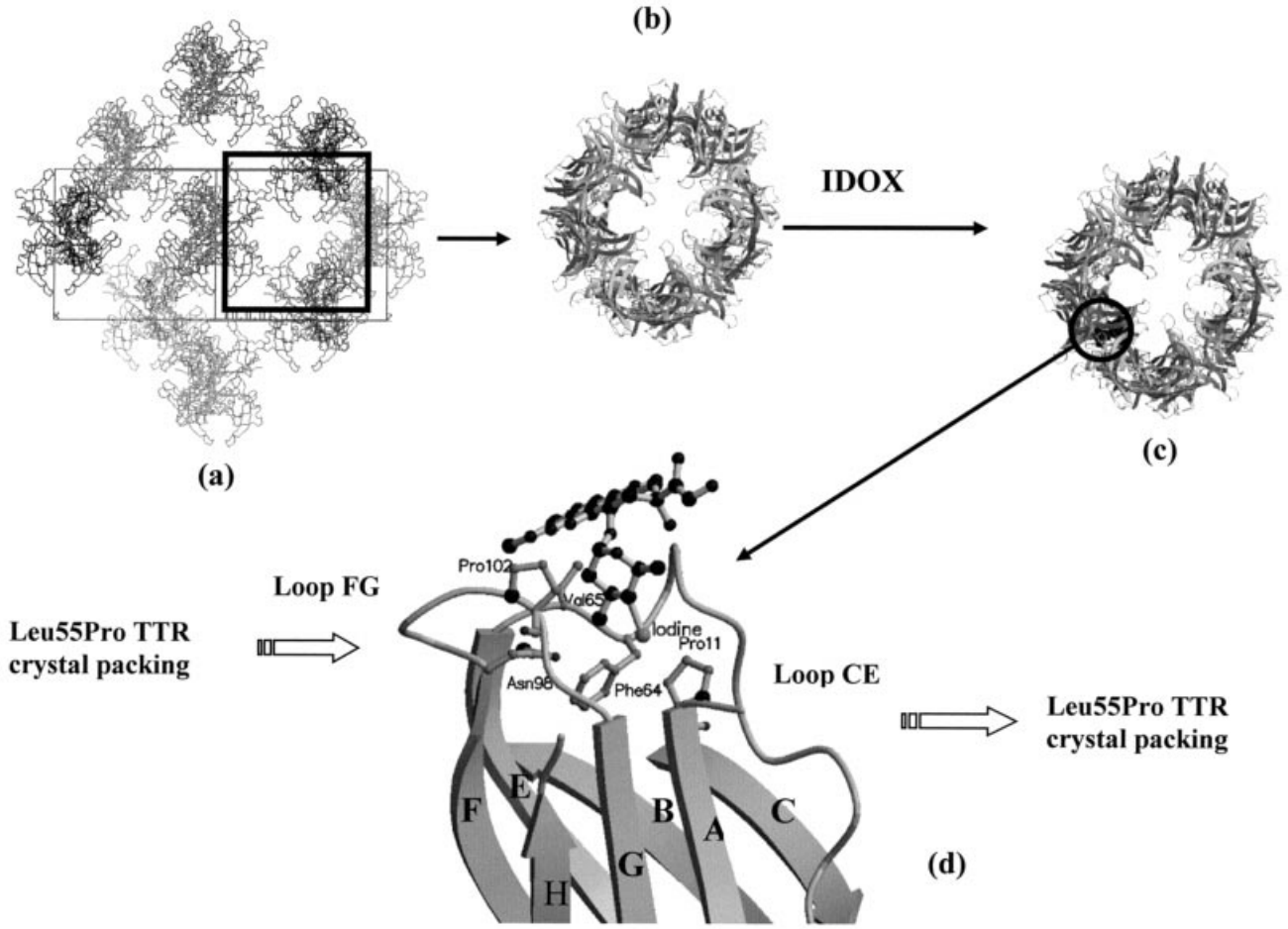

Figure 4 Leu-55Pro TTR–IDOX interaction

(**a**) Leu-55Pro TTR crystal unit cell. The *a*, *b* and *c* axes of the unit cell are indicated by solid lines (X, Y and Z, respectively). (**b**) Leu-55Pro TTR crystal tubular structure. (**c**, **d**) Leu-55Pro TTR–IDOX predicted model.

GRAMM. A high-resolution simulation was performed and the low-energy molecular solutions obtained were analysed with XtalView. The major cluster of ligand positions occurs around the first Leu-55Pro TTR–IDOX match (Figure 3). It has been observed that a more populated state is indicative of a preferential structure [22]. Thereby, the lowest-energy Leu-55Pro TTR–IDOX solution was chosen and further refined with LIGIN.

Figure 4(d) shows the final refined solution, with the halogenated amino sugar group inserted into a pocket between the DAGH and CBEF β-sheets and the long axis of the IDOX aromatic moiety nearly perpendicular to the direction of the β-sheets. In this Leu-55Pro TTR–IDOX complex the sugar amino group is H-bonded to the amide nitrogen of Glu-66 (2.97 Å) and to the carbonyl group of Asn-98 (2.98 Å), as listed in Table 1.

The sugar iodine atom forms a positive polar interaction with the main-chain carbonyl group of Arg-103 (3.51 Å) and it is close to the aromatic residue Phe-64 (Table 1 and Figure 4). The interaction between the iodine atom of IDOX and the benzene ring of Phe-64 is a notable feature of the Leu-55Pro TTR IDOX-binding site and might increase the drug affinity significantly. There is experimental evidence of favourable interactions between iodinated ligands and aromatic residues of proteins [23]. It seems plausible to propose that Phe-64 might be an important residue for IDOX binding in Leu-55Pro TTR and that an aromatic environment should be present in other proteins that bind the amyloid inhibitor IDOX, because that offers particularly favourable interactions with the iodine.

The IDOX chromophore ring makes positive hydrophobic contacts with residues Pro-102 and Val-65. It is also H-bonded to the Leu-55Pro TTR monomer by the main-chain carbonyl group of Glu-61.

The Leu-55Pro TTR–IDOX model, illustrated in Figure 4, explains why the Leu-55Pro TTR crystals are destroyed when soaked with IDOX. In fact, the Leu-55Pro TTR crystal structure revealed a strong interaction between monomers, leading to a tubular structure with an approx. diameter of 100 Å (Figure 4), the amyloid-like TTR oligomer. In this variant structure the main crystal-packing interactions are loop CE–loop CE, loop AB–α-helix and loop FG–loop FG. According to our molecular-modelling studies the IDOX-binding site corresponds to a region of interaction between monomers in the described tubular structure and disturbing these interactions results in disruption of the amyloid-like oligomer. Furthermore, this contact region is not present in the wild-type crystals, since they have different crystallographic packing interactions, and consequently the amyloid-like oligomer is not present. As a result, the wild-type

Table 1 Theoretical Leu-55Pro TTR–IDOX inter-atomic contacts

	Leu-55Pro TTR	IDOX
Hydrogen-bonding	Glu-61 CO	O-6 (2.97 Å); O-5 (3.37 Å)
	Glu-66 NH	N-3′ (2.97 Å)
	Asn-98 CO	N-3′ (2.98 Å)
	Asn-98 $N^{\delta 2}$	N-3′ (3.46 Å)
Van-der-Waals interaction	Arg-103 CO	I-4′ (3.51 Å)
Hydrophobic interaction	Pro-102 C^{γ}	C-1 (4.26 Å)
	Pro-102 C^{β}	C-2 (4.36 Å); C-3 (3.81 Å); C-4 (3.31 Å); C-5 (3.74 Å)
	Pro-102 C^{γ}	C-12 (4.32 Å); C-15 (3.90 Å)
	Pro-102 C^{β}	C-16 (3.40 Å)
	Pro-102 C^{γ}	C-17 (4.55 Å); C-18 (4.68 Å); C-21 (4.68 Å)
	Val-65 C^{α}	C-1′ (3.31 Å)
	Val-65 C^{β}	C-4′ (4.38 Å)
	Val-65 $C^{\gamma 1}$	C-6′ (4.78 Å)
	Glu-61 C^{δ}	C-21 (4.35 Å)
	Arg-103 C^{α}	C-21 (4.44 Å)
	Arg-104 C^{β}	C-21 (4.66 Å)
	Phe-64 $C^{\delta 2}$	I-4′ (4.13 Å, 4.6 Å)
	Phe-64 $C^{\epsilon 2}$	
	Pro-11 C^{β}	I-4′(3.89 Å, 3.14 Å)
	Pro-11 C^{δ}	

structure is not disturbed by the presence of the IDOX molecules, which do not bind wild-type TTR, as shown by our X-ray crystallographic studies.

Our results indicate that a TTR intermediate quaternary structure in the biochemical pathway that leads to amyloid deposition, which already binds the amyloid-binding drug IDOX, is isolated by the Leu-55Pro TTR crystallization procedure. This is corroborated by the fact that the Leu-55Pro TTR variant binds Thioflavin T, which is an amyloid marker (Figure 1). Biochemical studies of this mutant performed by Lashuel et al. [24] show that it aggregates at physiological conditions. Furthermore, this model is in agreement with our previous proposal about the molecular interactions observed in TTR amyloid protofibrils.

IDOX is being used as a therapeutic agent in AL patients and it has been reported to bind amyloid fibrils and induce their resorption. It is an anthracycline and this class of chemotherapeutic agents presents cardiotoxicity as the most important side effect. However, the results of several studies demonstrate that this compound is clearly less cardiotoxic than the parental drug doxorubicin, both in animal models [25–28] and in humans [29,30]. This compound was also tested in prion-infected hamsters and in this case clinical signs of the disease were delayed and survival time was prolonged [31]. Since IDOX is a chemotherapeutic agent, its use has been limited to AL amyloidosis, which is caused by a neoplastic plasma cell population in the bone marrow. Non-cytotoxic IDOX analogues have been investigated, but their access is restricted because of patent reasons (G. Merlini, personal communication).

All types of amyloid fibril have cross-β-pleated-sheet conformation and in this work we found that the iodinated sugar ring binds into a region between the two β-sheets of the Leu-55Pro TTR monomer, with the long axis of the IDOX aromatic moiety perpendicular to them. This interaction disturbs protein–protein interactions in the amyloid-like Leu-55Pro TTR structure, leading to its dissociation.

It has been reported that several tetracyclic compounds have amyloid-inhibitory properties in Alzheimer's disease [32] and that their effect is dependent upon an amino group and a hydrophobic cyclic moiety. These results are in agreement with our Leu-55Pro TTR–IDOX model in TTR amyloidosis, since there is a strong interaction of the IDOX amino group with Asn-98 and Glu-66, and the IDOX aromatic moiety makes hydrophobic contacts with the Leu-55Pro TTR amyloid-like structure (Table 1 and Figure 4).

Our results indicate that IDOX inhibits fibril formation by binding to TTR oligomers, in particular to the Leu-55Pro TTR amyloid-like structure, which was described previously by X-ray crystallography. Therefore, the present study is unique in providing a detailed molecular picture of the Leu-55Pro TTR–IDOX interaction, which might contribute to the development of non-cardiotoxic therapeutic agents for amyloidosis.

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