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Authors

Gasymov, Oktay K
Abduragimov, Adil R
Glasgow, Ben J

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Restoration of structural stability and ligand binding after removal of the conserved disulfide bond in tear lipocalin

Oktay K. Gasymov[*], **Adil R. Abduragimov**, and **Ben J. Glasgow**[*]
Departments of Pathology and Ophthalmology and Jules Stein Eye Institute, University California at Los Angeles, CA 90095

Abstract

Disulfide bonds play diverse structural and functional roles in proteins. In tear lipocalin (TL), the conserved sole disulfide bond regulates stability and ligand binding. Probing protein structure often involves thiol selective labeling for which removal of the disulfide bonds may be necessary. Loss of the disulfide bond may destabilize the protein so strategies to retain the native state are needed. Several approaches were tested to regain the native conformational state in the disulfide-less protein. These included the addition of thrimethylamine N-oxide (TMAO) and the substitution of the Cys residues of disulfide bond with residues that can either form a potential salt bridge or others that can create a hydrophobic interaction. TMAO stabilized the protein relaxed by removal of the disulfide bond. In the disulfide-less mutants of TL, 1.0 M TMAO increased the free energy change (ΔG^0) significantly from 2.1 to 3.8 kcal/mol. Moderate recovery was observed for the ligand binding tested with NBD-cholesterol. Because the disulfide bond of TL is solvent exposed, the substitution of the disulfide bond with a potential salt bridge or hydrophobic interaction did not stabilize the protein. This approach should work for buried disulfide bonds. However, for proteins with solvent exposed disulfide bonds, the use of TMAO may be an excellent strategy to restore the native conformational states in disulfide-less analogs of the proteins.

Keywords

TMAO; urea unfolding; protein destabilization; ligand binding kinetics; disulfide bond

1. Introduction

Many proteins use disulfide bonds to regulate their stabilities and functions. Protein folding kinetics are also greatly influenced by disulfide bond formation [1]. To investigate dynamics

[*]Corresponding authors: Oktay K. Gasymov, 100 Stein Plaza, Rm# B267, Los Angeles, CA 90095. Phone: (310) 825-6261; Fax: (310) 794-2144; ogassymov@mednet.ucla.edu Ben J. Glasgow, 100 Stein Plaza, Rm# B269, Los Angeles, CA 90095. Phone: (310) 825-6998; Fax: (310) 794-2144; bglasgow@mednet.ucla.edu.

Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version.

of proteins, some spectroscopic techniques, such as site-directed spin labeling (SDSL) use labeled Cys as a “reporter group”. To avoid disulfide scrambling or mislabeling other native or introduced Cys residues of expressed proteins removal of the native disulfide bond (or bonds) may be necessary. Removal of disulfide bonds alone produces variable functional effects. For example in ribonuclease T1 (RNase T1), reduction and carboxyamidation significantly destabilize the protein [2], which at 20°C 73% becomes unfolded. In another protein tear lipocalin, TL, the ligand binding properties are greatly influenced by the single disulfide elimination [3-6]. Secondary structural content of reduced TL is not modified compared to that of native TL. However, relaxation of structure induced by disulfide removal increases binding affinity for retinol [3] and retinoic acid [4]. Ligand binding kinetics are also altered by removal of the disulfide bond. In disulfide-less TL, the time constant for the binding of a bulky ligand (NBD-cholesterol) is decreased about 4 fold. Only a moderate decrease (1.5 fold) is observed for the NBD-labeled phospholipid [5]. A natural reagent that promotes formation and stabilizes the native fold could aid the use of disulfide-less proteins to study and expand applicability of solution structure techniques that require Cys labeling techniques.

The osmolyte, thrimethylamine N-oxide (TMAO), is known to stabilize proteins against denaturating agents, e.g., urea [7-11]. Some marine organisms use TMAO to stabilize proteins in conditions where intracellular urea concentrations are elevated [11]. TMAO stabilizes the native protein fold to induce folding by preferential exclusion from the immediate vicinity of both the native and denaturated states of the protein [9-11].

Abundant data are available for use of TMAO in denaturant titrating experiments [11-14]. However, only two studies have investigated the influence of TMAO on ligand binding. One used TMAO to change the conformational state of muscle glycogen phosphorylase *b* to study flavin adenine dinucleotide binding [15]. The other study found that TMAO induces folding in the CXC chemokine receptor 1 N-domain and promotes binding to the chemokine interleukin 8 with higher affinity [16]. Investigation of the impact of TMAO on a protein relaxed by removal of disulfide bond with restoration of binding to small ligands such as lipids and drugs is needed. In this study tear lipocalin (TL) was selected for experiments because the ligand binding of TL has been well characterized. The demonstrated ligands include fatty acids of varying lengths, phospholipids, alkyl alcohols, glycolipids, as well as various synthetic ligands and drugs [17-21]. Comprehensive information about structure, dynamics and function is also available for TL. The solution and crystal structures of TL have been determined by site-directed tryptophan fluorescence (SDTF) and X-ray crystallography [22,23]. TL possesses the classic lipocalin fold, i.e. eight anti-parallel β-strands with +1 topology [24]. In the current study, the lipocalin fold of TL was destabilized by removal of the conserved disulfide bond. The impact of TMAO on ligand binding of disulfide-less TL was tested. In addition the disulfide bond of TL was replaced with residues that form either a salt bridge or hydrophobic interaction to test recovery of stability of the protein to that of native form.

Beside of its mechanistic significance, this study also has practical importance. Here, we show that TMAO may recover both structural stability and ligand binding properties.

2. Materials and methods

2.1. Materials

Urea, thrimethylamine N-oxide and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-Ol (NBD cholesterol), was purchased from Invitrogen (Carlsbad, CA).

2.2. Site-directed mutagenesis and plasmid construction

The TL cDNA in PCR II (Invitrogen), previously synthesized (21), was used as a template to clone the TL gene spanning bases 115–592 of the previously published sequence [25] into pET 20b (Novagen, Madison, WI). Flanking restriction sites for NdeI and BamHI were added to produce the major isoform of the native protein sequence as found in tears with the addition of an initiating methionine [26]. To construct mutant proteins, the previously well characterized TL mutant, C101L, was prepared with oligonucleotides (Universal DNA Inc., Tigard, OR) by sequential PCR steps [27]. Using this mutant as a template, mutant cDNAs were constructed in which selected amino acids were additionally substituted with the desired amino acid. C101L (denoted as TL) was used as a template because it exhibited very similar structural features and ligand binding characteristics as those of the native protein [28]. In urea denaturation experiments, the use of C101L as a template prevents the possibility of intramolecular disulfide scrambling in the mutants where either residue 61 or 153 is substituted. The mutants of TL, which lack the disulfide bond, include C101L/C61S (for simplicity denoted as S61), C101L/C153S (S153) and C101L/C61S/ C153S (S61/S153). The mutant C61E/ C153R (E61R153) was constructed to test if potential salt bridge between residues 61 and 153 could simulate native disulfide bridge between these residues. The mutant C61A/C153A (A61A153) was constructed to test if substitution of the disulfide bond with a small hydrophobic residue could stabilize the protein.

2.3. Expression and purification of mutant proteins

The mutant plasmids were transformed in E. Coli, BL 21 (DE3). Cells were cultured and proteins were expressed, purified, and analyzed as described elsewhere [22,29].

The protein concentrations of stock and dilute solutions were determined by using the molar extinction coefficient of TL (ε_{280} = 13760 $M^{-1}cm^{-1}$ [30].

2.4. CD spectral measurements

CD spectra were recorded at room temperature on a Jasco J-810 spectropolarimeter. Far-UV spectra were recorded using path lengths of 0.1 mm. The concentrations of the proteins were about 1.8 mg/ml. Each CD spectrum represents the average of nine scans. CD spectra were recorded in mdegrees and then converted to $deg{\cdot}cm^2{\cdot}dmol^{-1}$.

2.5. Urea equilibrium unfolding with and without TMAO

In equilibrium unfolding measurements, TL and mutant proteins, 1.8 mg/ml, were incubated with various concentrations of urea with and without 1.0 M TMAO at room temperature for at least 18 h. Circular dichroic spectra were measured at room temperature. The fractions of unfolded protein at each urea concentration were calculated from CD data at 217 nm as Y =

$(\theta_N - \theta) / (\theta_N \theta - \theta_D)$. θ is observed ellipticity; n and u refer to the native and unfolded states, respectively. The denaturation data were fit to the following equation derived for two-state model by the nonlinear least-squares method using OriginPro version 8 (OriginLab Corp., Northampton, MA).

$$Y = \frac{y_N + m_N\,[urea] + (y_D + m_D\,[urea])\,exp - \frac{\Delta G^0 - m[urea]}{RT}}{1 + exp - \frac{\Delta G^0 - m[urea]}{RT}}$$

where, y_N and y_D are the fractions of unfolded proteins at 0 M and 10 M urea, respectively. ΔG^0 is the free energy change in the absence of denaturant assuming linear dependence of ΔG on urea concentration, m_N and m_D are the slopes of the baselines before and after the transition, respectively, m is the rate of change of free energy as a function of denaturant concentration.

2.6. Time dependent binding kinetics by steady-state fluorescence

Time dependent binding studies were performed NBD cholesterol (2 μM) in 10 mM sodium phosphate buffer at pH 7.3 with and without 1.0 M TMAO. The stock solution of the protein (mutant S61/S153) was added to the ligand solutions while stirred. The final concentration of the protein was 0.2 μM. Each experiment was performed at least 3 times. The excitation and emission λ were 410 nm and 527 nm, respectively. The excitation and emission bandwidths were 2 nm and 4 nm, respectively. The time dependent kinetic curves were analyzed with a single exponential function, $y=y0+A(1-\exp(-t/\tau))$, where y0, A and τ are constant, amplitude of exponential increase and time constant, respectively. The nonlinear least-squares method was used for the fitting of the data with OriginPro 8 (OriginLab Corp., Northampton, MA).

3. Results and Discussion

3.1 Impact of TMAO on the lipocalin fold destabilized by removal of the conserved disulfide bond

In this study the influence of TMAO on structure was assessed after tear lipocalin was destabilized by removal of the conserved disulfide bond. TMAO protects from urea induced unfolding of the protein. Figure 1 shows the representative far-UV CD spectra of S61S153 with and without 1.0 M TMAO at various concentrations of urea. The CD spectrum of the mutant S61S153 without TMAO shows almost fully unfolded state at 5.0 M urea (Figure 1). However in the presence of 1.0 M TMAO and 5.0 M urea the far-UV spectrum of S61S153 indicates the presence of significant secondary structure in the protein. In the absence of urea, the far-UV CD spectra of TL with and without disulfide bond are almost identical. Secondary structural content is the same for both proteins [5]. TMAO stabilizes the lipocalin fold destabilized by removal of the conserved disulfide bond. Urea denaturation curves for this mutant are shown in Figure 2. The mutant S61S153 with 1.0 M TMAO shows a significant shift in the midpoint of transition. The addition of 1.0 M TMAO increases the free energy change (ΔG^0) significantly from 2.1 (reported previously in [5] to 3.8 kcal/mol (Table 1). ΔG^0 value of the mutant S153 in the presence of 1.0 M TMAO is significantly

increased to 4.9 kcal/mol (Table 1, Fig. 2). Small differences in behavior of these disulfide-less mutants were also noticed previously [5]. Use of 1.0 M TMAO did not change significantly m-values of transitions consistent with previous findings (Table 1). The stabilization effect of 1.0 M TMAO in the disulfide-less mutants with various residue substitutions is very similar (Table 1).

The widely accepted mechanism of stabilization of native protein folds in the presence of denaturing agents is the preferential exclusion of TMAO molecules from vicinities of native and unfolded states [9-11]. The stabilization effect of TMAO on a protein is manifested by producing a shift of the midpoint of transitions to higher denaturant concentration. The effects of disulfide bonds on proteins are not uniform, but protein specific. In TL, removal of the conserved single disulfide bond not only decreases stability but also significantly alters ligand binding properties. Previously, it has been shown that the disulfide bond modulates ligand binding of TL [5]. The regulation of ligand binding by the disulfide bond may have functional significance [4]. Thioredoxin and tear lipocalin, both of which are present in human prostate, interact with each other. Thioredoxin reduces the disulfide bond of TL and changes ligand binding properties. The impact of thrioredoxin to TL is similar to that of small disulfide reducing agents, such as β-mercaptoethanol [4]. It is plausible that reduced TL may have a functional role in human prostate. Structural characterization of reduced TL is relevant.

Thus, TMAO is not just a stabilizer against denaturating agents, but also stabilizes relaxed proteins (not unfolded) by removal disulfide bond. TMAO has been shown to promotes disulfide formation in a model system and may play important role in biological system [31]. Indeed, reductive unfolding rate of onconase is diminished in the presence of TMAO [32]. Use of mutations to disrupt the disulfide bond excludes stabilization of the protein by disulfide bond formation promoted by TMAO. Therefore, in our system TMAO assessed as osmolyte excluding its disulfide formation property.

3.2 Substitution Cys residues of the disulfide bond with a potential salt bridge

Replacement of the Cys residues 61 and 153, with those that can form a salt bridge (mutant E61R153) helps determine the exposure of the disulfide bond. A disulfide in which residues are exposed to solvent is less likely to form a salt bridge. Stability of the protein was tested with a urea titration experiment. Figure S1 shows the far-UV CD spectra of E61R153 at various urea concentrations. In the absence of urea, the far-UV CD spectrum is very similar to that of native as well as other disulfide-less TL mutants. The addition of urea to mutant E61R153 results in a progressive loss of secondary structure of the protein (Figure S1). At 9.5 M urea, the CD spectrum shows complete elimination of secondary structure. Qualitatively similar CD spectra were obtained for the other proteins at various urea concentrations (For example, Figure S2). Urea denaturation curves for TL and mutants that lack of disulfide bond are shown in Figure 2. Denaturation curves of TL and the mutant S153, published previously [5], are shown for as a point of reference only. Removing the disulfide bond decreases the free energy change (ΔG^0) significantly from 4.9 to 2.3 kcal/mol (Table 1). Substitution of disulfide bond (Cys61-Cys153) with possible salt bridge (E61 R153) did not stabilize the protein (Table 1). Salt bridges in protein contribute to stability

differently [33,34]. Due to unfavorable desolvation, the surface salt bridges exposed to solvent contribute very little to protein stability [33]. Most likely, the residues of the disulfide bond, Cys61 and Cys153, are solvent exposed congruous with the observation that the conserved disulfide bond is accessible to the protein thioredoxin [4], and, therefore, to the solvent.

3.3 Substitution Cys residues of the disulfide bond with Ala residues for the potential hydrophobic interaction

Another approach was also assessed to mimic the native structural packing in disulfide-less mutant of TL. Both Cys residues at positions 61 and 153 were substituted with Ala residues, which have the smallest hydrophobic side chain. Urea titration experiment indicates that possible hydrophobic packing for these residues was not achieved. Free energy change (ΔG^0) for the mutant A61A153 is similar to that of other disulfide-less mutants of TL (Figures S2 and 3 and Table 1). Addition of 1.0 M TMAO to the A61A153 accompanied stabilization of the protein to the same degree (from 2.0 to 3.7 kcal/mol, Table 1).Thus, stabilization was not noticed in the mutant (A61A153), which have smallest hydrophobic groups. The result is also consistent with the notion that the disulfide bond is solvent exposed. In the case of A61A153, a putative favorable entropic effect is not enough for hydrophobic interaction between solvent accessible Ala residues.

3.4 Restoration of ligand binding in a disulfide-less mutant

Figure 4 shows time dependent NBD-cholesterol binding to S61S153 with and without 1.0 M TMAO. The time constant of the ligand binding to S61S153 is about 3.2 s consistent with previous findings. S61S153, in the presence of 1.0 M TMAO, shows a moderate increase in the time constant to 5.2 s. However, this value is significantly less from time constant (11.8 s) of NBD-cholesterol binding to native TL (Figure 4).

Stabilization of the protein fold by TMAO does not necessarily mean that native conformational states are recovered. Previously, NBD-cholesterol was used to characterize the influence of TMAO to the disulfide-less mutants of TL. Similar time constants of ligand binding kinetics were revealed for NBD-C12-HPC (2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl- sn-glycero-3-phosphocholine) to TL with and without the disulfide [5]. However, NBD-cholesterol shows significantly different time constants for these forms of TL. Relaxation of the structure by removal of disulfide bond decreases the time constant of ligand binding to 3.3 s from 11.8s. For that reason, NBD-cholesterol binding kinetics is a good indicator of the conformational state of TL. The more rapid ligand binding the disulfide-less mutation is diminished by TMAO as the native conformation begins to reform. TMAO significantly stabilizes structure of disulfide-less TL, but does not fully re-establish ligand binding properties. These findings may have significant implications. Modulation of protein functions induced by reduction of disulfide bonds are abrogated in the presence of TMAO. In the cell TMAO stabilizes the structure of proteins but does not change the functions regulated by the redox system.

The major contributions from our data are: 1. TMAO restores protein stability relaxed by removal of a conserved disulfide bond. 1.0 M TMAO almost fully restores stability of the

lipocalin fold. 2. The concentration of TMAO should be matched to individual proteins for maximum restoration of the native state destabilized by removal of a disulfide bond (or bonds). TMAO allows ligand binding to be assessed in a disulfide-less protein. 4. TMAO allows the use of thiol selective spectroscopic labels in a disulfide-less proteins. 5. If the disulfide is solvent exposed, then salt bridge formation or hydrophobic interaction cannot be achieved by replacement with the relevant amino acids for the disulfide bond. In this situation the use of TMAO may be the best method to restore stability in cysteine substituted proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CD	circular dichroism
NBD cholesterol	22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-Ol
RNase T1	ribonuclease T1
SDSL	site-directed spin labeling
TL	tear lipocalin
TMAO	thrimethylamine N-oxide

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Highlights

- Instability from removal of the conserved disulfide bond in tear lipocalin is reversed by TMAO.
- Ligand binding is partially restored by TMAO in the disulfide-less protein.
- TMAO is preferred for stabilization where solvent exposed disulfides are removed.

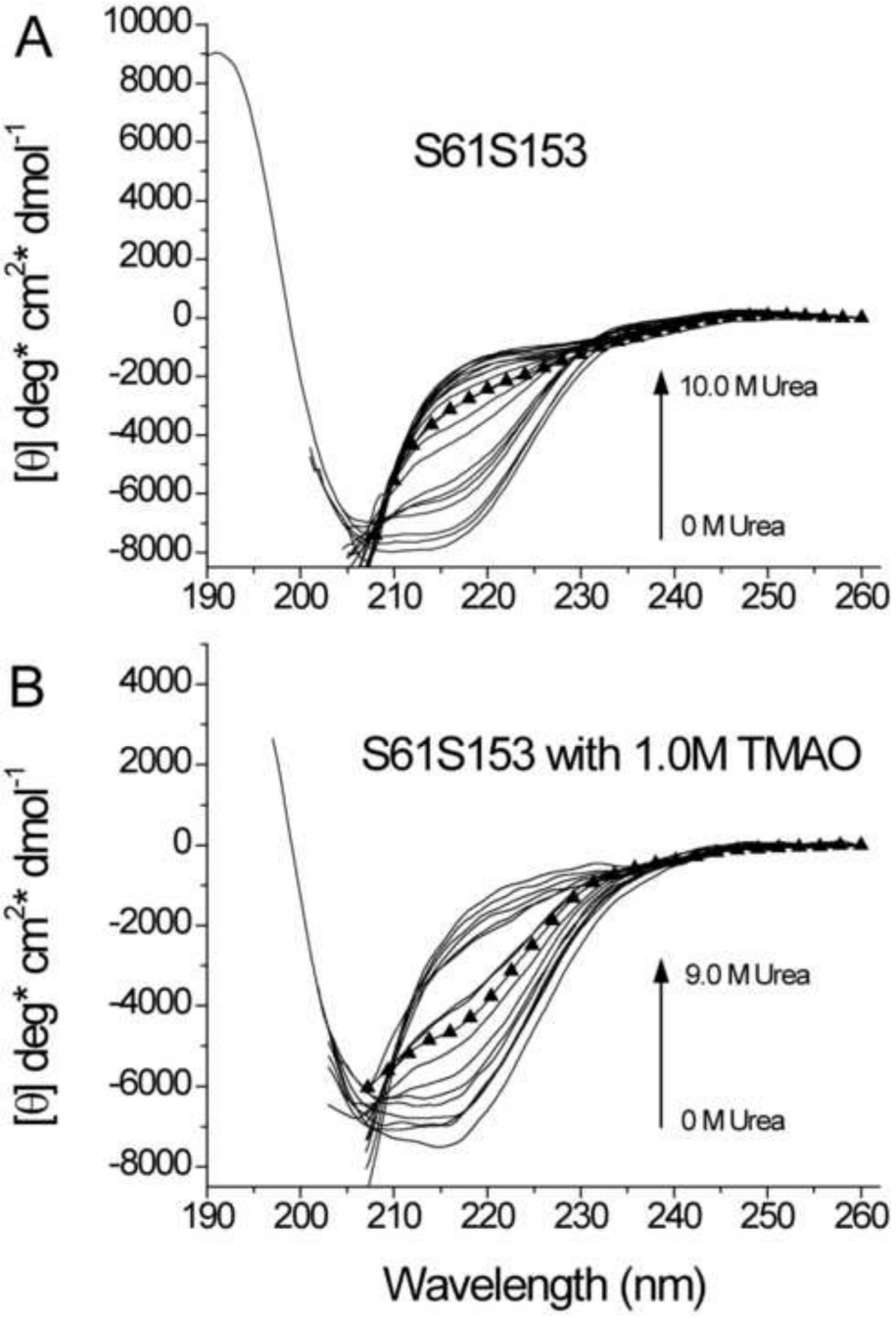

Figure 1.

Representative far-UV CD spectra for the mutant S61S153 without (A) and with (B) 1.0M TMAO at various urea concentrations. For each series of experiment the spectrum at 5.0 M urea allocated with triangular solid symbols

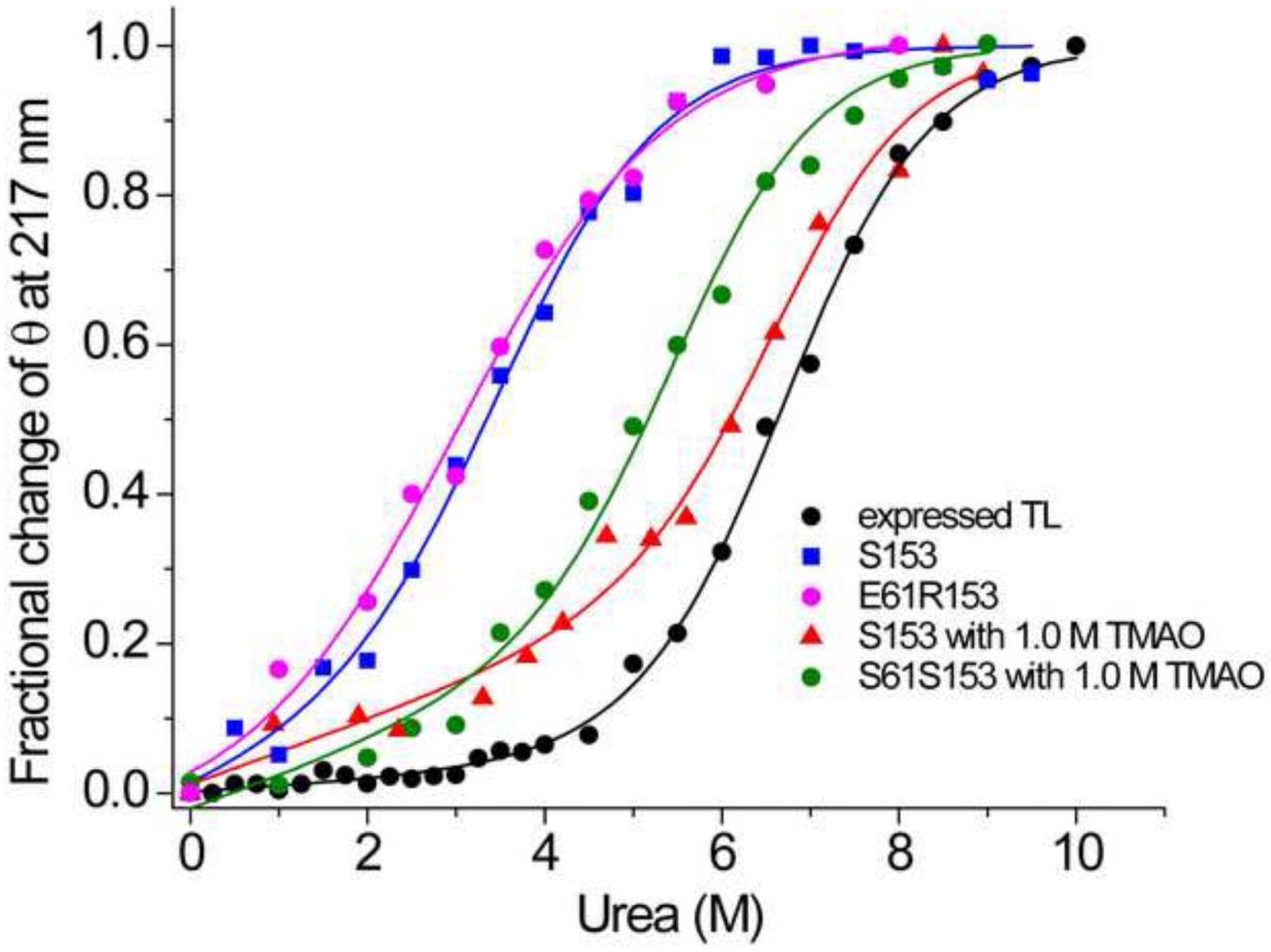

Figure 2.

Urea denaturation curves for TL and its disulfide-less mutants with and without TMAO. The symbols denote the fractional changes for the proteins at various urea concentrations calculated from respective far-UV CD data at 217 nm. The solid cures are the best fit of the data to two state transition model (see Materials and Methods). The recovered parameters are shown in Table 1.

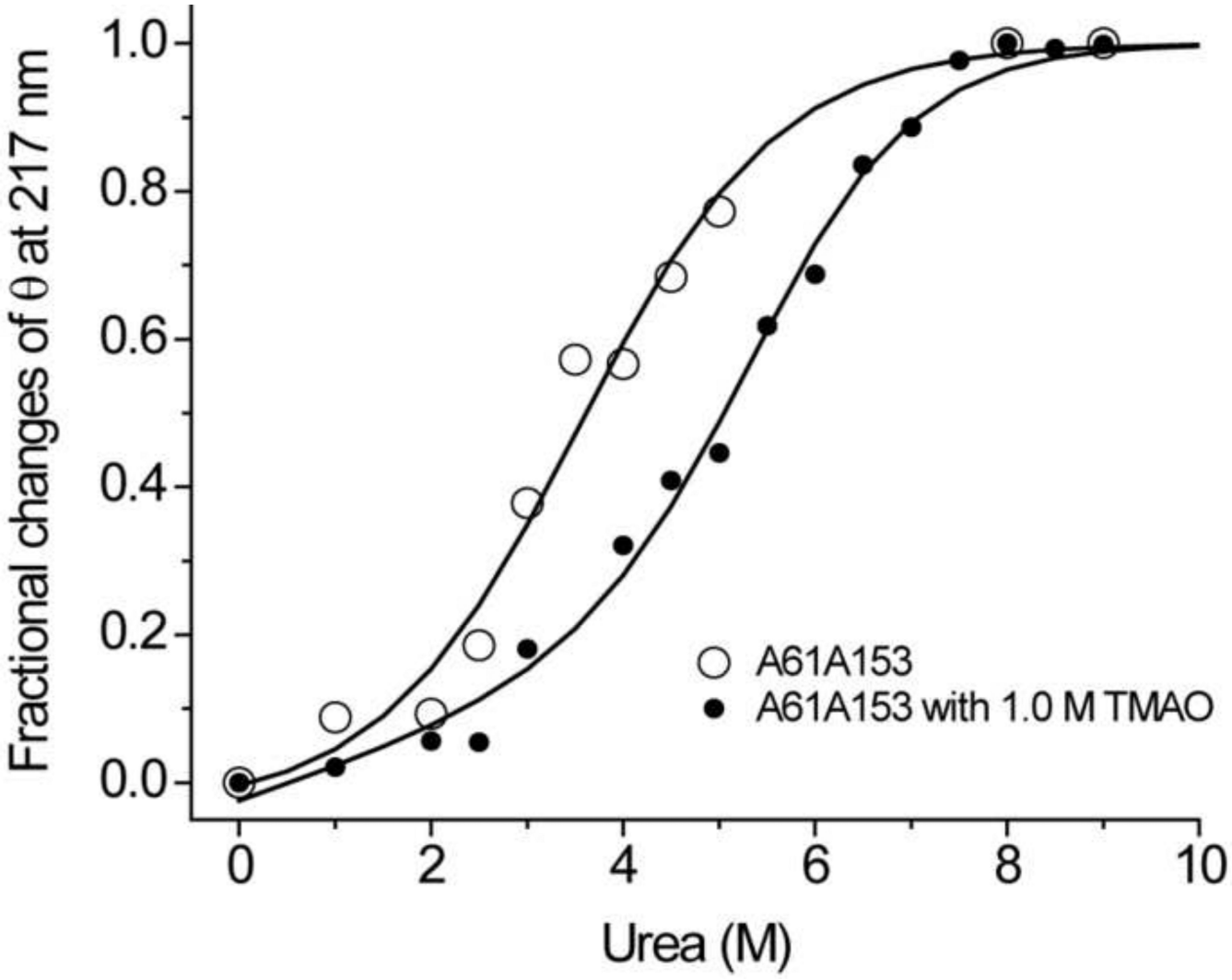

Figure 3.

Urea denaturation curves for the mutant A61A153 with and without TMAO. The symbols denote the fractional changes for the proteins at various urea concentrations calculated from respective far-UV CD data at 217 nm. The solid cures are the best fit of the data to two state transition model (see Materials and Methods). The recovered parameters are shown in Table 1.

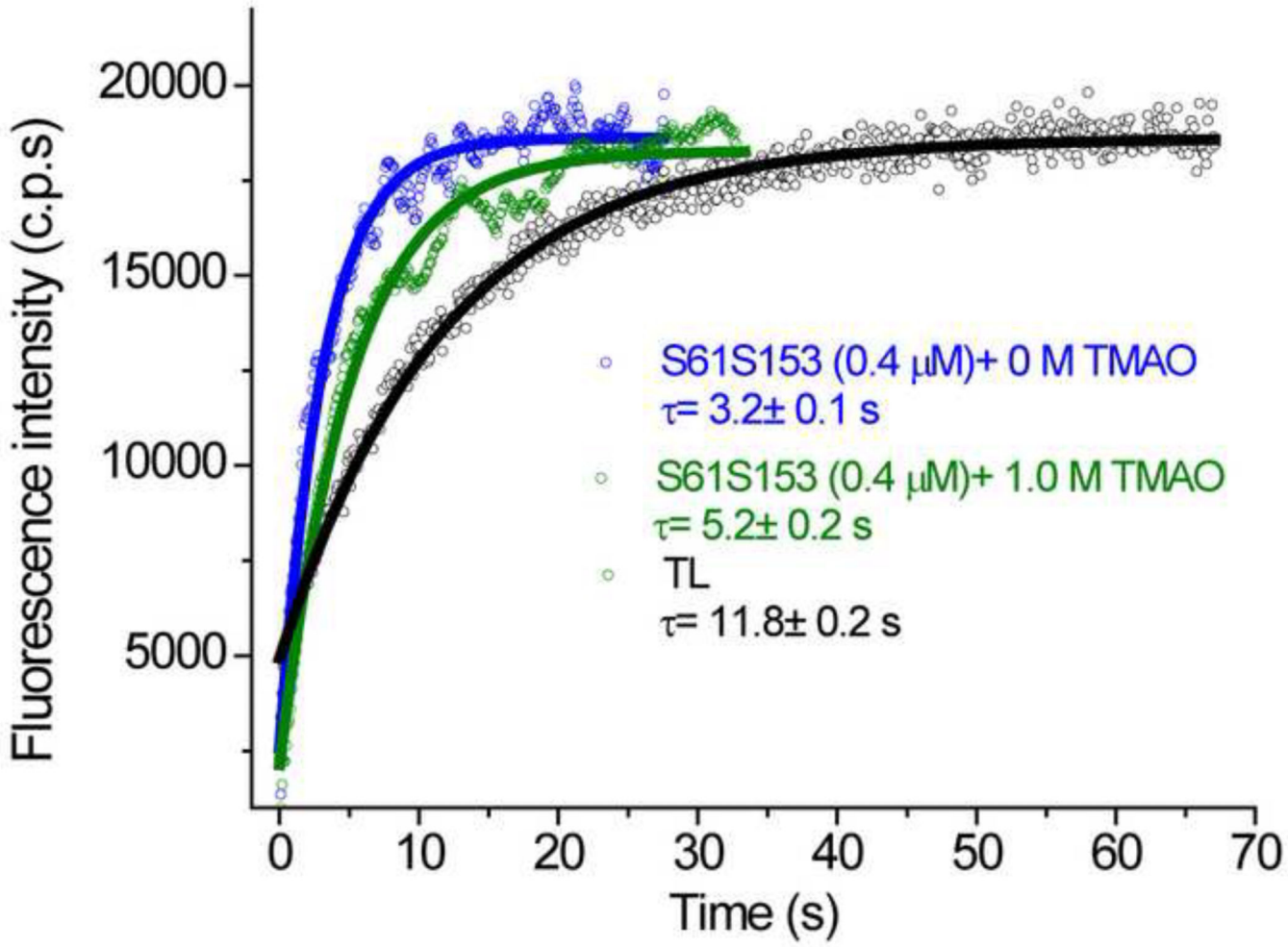

Figure 4.

Influence of TMAO to ligand binding kinetics of TL destabilized by removal of the conserved disulfide bond. Green and blue symbols (average of 3 separate experiments) represent time dependent binding of NBD-cholesterol to S61/S153 with and without TMAO, respectively. Black symbols represent data for TL with intact disulfide bond. Solid curves represent the best fit of the data to single exponential functions. Data for TL, which was reported previously [5], were added to show extent of recovery in the ligand binding.

Table 1

Influence of TMAO to urea unfolding parameters for TL and its disulfide-less mutants

Protein	ΔG (kcal mol^{-1})	m (kcal mol^{-1} M^{-1})
recombinant TL	4.9± 0.2	0.72± 0.03
S153	2.3± 0.5	0.64± 0.08
E61R153	1.5± 0.3	0.50± 0.07
A61A153	2.0± 0.4	0.57± 0.04
S61S153 with 1.0 M TMAO	3.8± 0.3	0.69± 0.05
S153 with 1.0 M TMAO	4.9± 1.0	0.73± 0.13
A61A153 with 1.0 M TMAO	3.7± 0.6	0.68± 0.09