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The conserved disulfide bond of human tear lipocalin modulates conformation and lipid binding in a ligand selective manner

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

Abstract

The primary aim of this study is the elucidation of the mechanism of disulfide induced alteration of ligand binding in human tear lipocalin (TL). Disulfide bonds may act as dynamic scaffolds to regulate conformational changes that alter protein function including receptor-ligand interactions. A single disulfide bond, (Cys61-Cys153), exists in TL that is highly conserved in the lipocalin superfamily. Circular dichroism and fluorescence spectroscopies were applied to investigate the mechanism by which disulfide bond removal effects protein stability, dynamics and ligand binding properties. Although the secondary structure is not altered by disulfide elimination, TL shows decreased stability against urea denaturation. Free energy change (ΔG^0) decreases from 4.9 ± 0.2 to 2.1 ± 0.3 kcal/mol with removal of the disulfide bond. Furthermore, ligand binding properties of TL without the disulfide vary according to the type of ligand. The binding of a bulky ligand, NBD-cholesterol, has a decreased time constant (from 11.8 ± 0.2 to 3.3 s). In contrast, the NBD-labeled phospholipid shows a moderate decrease in the time constant for binding, from 33.2 ± 0.2 to 22.2 ± 0.4 s. FRET experiments indicate that the hairpin CD is directly involved in modulation of both ligand binding and flexibility of TL. In TL complexed with palmitic acid (PA-TL), the distance between the residues 62 of strand D and 81 of loop EF is decreased by disulfide bond reduction. Consequently, removal of the disulfide bond boosts flexibility of the protein to reach a CD-EF loop distance (24.3 Å, between residues 62 and 81), which is not accessible for the protein with an intact disulfide bond (26.2 Å). The results suggest that enhanced flexibility of the protein promotes a faster accommodation of the ligand inside the cavity and energetically favorable ligand-protein complex.

Keywords

excited protein states; ligand binding; tear lipocalin; protein dynamics; disulfide motive; FRET; time-resolved fluorescence

1. Introduction

Human tear lipocalin (TL), alias von Ebner's gland protein, belongs to the lipocalin protein family whose membership extends to both eukaryotes and prokaryotes. The solution

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*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.

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structure of TL was determined by site-directed tryptophan fluorescence (SDTF). TL has the typical lipocalin fold, which consists of eight antiparallel β -strands with a repeated +1 topology [1]. X-ray crystallography of TL is concordant with the solution structure [2].

TL has unusually diverse functions distinct from the other members of the lipocalin family. TL is implicated in scavenging lipid from the corneal surface to prevent the formation of lipid induced dry spots [3], solubilization of lipid in tears [1], antimicrobial activity [4, 5], cysteine proteinase inhibition [6], transport of lipid molecules in saliva [7], transport of retinol in tears [8], scavenging potentially harmful lipid oxidation products [9], transport of antioxidants in tears [10], and endonuclease activity [11]. In tears, TL is the principal ligand binding protein. An assortment of fatty acids, alkyl alcohols, glycolipids, phospholipids and cholesterol have been extracted from native TL [12]. In addition to these endogenous ligands, TL binds to various synthetic ligands [12–15]. TL may stabilize the tear film by interaction with and subsequent incorporation into the superficial lipid layer [3, 16–18]. All above-mentioned functions fit the theme that TL acts as an extracellular lipid chaperone to modulate lipid trafficking, distribution and clearance in the ocular surface interfaces.

Implicit to understanding the functions of TL is the elucidation of ligand binding mechanisms. Fatty acids are the most investigated class of lipid ligands for TL. Bound fatty acid does not have a unique position in the cavity of TL, but instead is distributed asymmetrically [19]. In ligand binding, the side chain dynamics of TL are also altered in an asymmetric fashion. Hence, the intracavitary dynamics of fatty acids are not stochastic. The excited protein state is an active conformation of the protein in ligand binding and consists of many substates. The protonation state of Glu 27 in loop AB sets the ligand bound conformation of TL [20, 21]. Loop AB as well as hairpins GH and CD exhibits larger changes compared with other parts of the protein in ligand binding. Strand D of TL, therefore, the hairpin CD, is linked to the C-terminus of the protein by a single disulfide bond and constrains conformational freedom. Cys61-Cys153 is the only disulfide bond of TL and is highly conserved in the lipocalin family. Evidence suggests a role in both structural stabilization and ligand binding [22]. Human thioredoxin enzymatically reduces TL's disulfide bond [23]. Both chemical and enzymatic reductions of the disulfide bond have induced an identical increase in the binding affinity of retinoic acid to TL [23]. The molecular mechanism by which the conserved disulfide bond regulates the ligand binding is unknown. However, a clue may exist in both SDTF solution and X-ray crystal structure studies that indicate that the hairpin CD is involved in ligand binding [19, 20]. One possibility is that the reduction of the disulfide bond may liberate the hairpin CD and enhance the protein plasticity, therefore, directly influence ligand binding.

Here, circular dichroism and fluorescence spectroscopies were applied to investigate changes in the protein stability, dynamics and ligand binding properties upon disulfide bond removal. Particularly, the mechanism by which disulfide bond reduction affects the dynamics of the hairpin CD of TL is investigated by monitoring excursions between the loops CD and EF. Selective response of TL to ligand binding by disulfide bond reduction has physiological implications.

2. Materials and methods

2.1 Materials

Palmitic acid, urea, N-acetyl-L-tryptophanamide (NATA), guanidine hydrochloride (GdnHCl) and other chemicals used to prepare various buffers were purchased from Sigma-Aldrich (St. Louis, MO). 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS), 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholesterol (NBD cholesterol), 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-

yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD C₁₂-HPC) and 11-((5-dimethylaminonaphthalene-1-sulfonyl)amino)undecanoic acid (DAUDA) were purchased from Invitrogen (Carlsbad, CA). Tris(2-Carboxyethyl) phosphine hydrochloride (TCEP) was obtained from Thermo Fisher Scientific Inc. (Rockford, IL).

2.2 Site-directed mutagenesis and plasmid construction

The TL cDNA in PCR II (Invitrogen), previously synthesized [24], was used as a template to clone the TL gene spanning bases 115–592 of the previously published sequence [8] 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 [25]. To construct mutant proteins, the previously well characterized TL mutant, C101L, was prepared with oligonucleotides (Universal DNA Inc., Tigard, OR) by sequential PCR steps [26]. Using this mutant as a template, mutant cDNAs were constructed in which selected amino acids were additionally substituted with the desired amino acid (Ser, Cys or Trp). The amino-terminal residue His1 corresponds to the bases 115–118 according to Redl [8]. Consequently, the same register was used for numbering the amino acids in TL.

To examine the influence of the conserved disulfide bond (Cys61-Cys153) of TL on protein stability and ligand binding characteristics, several TL mutants were constructed. 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 [27]. It is noteworthy that Cys101 was substituted with Ser in crystallographic studies of both apo- and holo-forms of TL [2, 20]. 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 W62C81 has been characterized previously [28], and was used in FRET experiments.

2.3 Expression and Purification of Mutant Proteins

The plasmids corresponding to each mutant were transformed in *E. coli* BL21(DE3), and cells were cultured according to the manufacturer's protocol (Novagene). Cell lysis was performed as described [29]. The supernatant was treated with methanol (40% final concentration) at 4 °C for 2.5 h and the resulting suspension was centrifuged at 3000g for 30 min. The dialysis was conducted against 50 mM Tris-HCl, pH 8.4, at 4 °C. The dialysate was treated with ammonium sulfate 45–75% saturation. The resulting precipitate was dissolved in 50 mM Tris-HCl, pH 8.4, and applied to a Sephadex G-100 column (2.5×100 cm) equilibrated with 50 mM Tris-HCl, 100 mM NaCl, pH 8.4. The fraction containing the mutant protein was dialyzed against 50 mM Tris-HCl, pH 8.4, and applied to a DEAE Sephadex A-25 column. Bound protein was eluted with a 0–0.8 M NaCl gradient. Eluted fractions containing mutant proteins were centrifugally concentrated (Amicon, Centricon-10). The purity of mutant proteins was verified by SDS-tricine gel electrophoresis [12].

The protein concentrations of stock and dilute solutions were determined by the biuret and the Lowry methods, respectively [30, 31] and were confirmed by the calculated molar extinction coefficient of TL. For comparison with prior crystallographic studies and to avoid complications of delipidation (see 3.1 below), expressed TL (xp-TL) without further delipidation was used in this study. Holo-proteins were obtained by saturation of the expressed proteins with palmitic acid (1:3). For clarity, the xp-TL complexed with palmitic acid (PA) is abbreviated as PA-TL.

2.4 Fluorescence labeling of TL mutant

The TL mutant W62C81 was labeled using a 15 fold molar excess of 1,5-IAEDANS in buffer 10 mM sodium phosphate, pH 7.3 at 4 °C overnight. Free label was removed by gel filtration on a desalting column (Pharmacia Biotech HiTrap, 5 mL). The labeling efficiency was calculated using the molar extinction coefficient $\epsilon_{336} = 5700 \text{ M}^{-1} \text{ cm}^{-1}$ for 1,5-IAEDANS. The protein concentration was determined by the biuret and Lowry (for dilute solution) methods. The efficiency of labeling for W62C81 was 0.83. In designated FRET experiments, TCEP was used in 20 fold molar excess of the protein to reduce the native disulfide bond (residues 61 and 153). The protein was incubated with TCEP for about 1 hour before measurement.

2.5 Absorption Spectroscopy

UV absorption spectra were recorded at room temperature using a Shimadzu UV-2400PC spectrophotometer. All experiments in this work were performed in 10 mM sodium phosphate, pH 7.3.

2.6 CD spectral measurements

CD spectra were recorded at room temperature on a Jasco J-810 spectropolarimeter. Path lengths of 0.2 mm and 10 mm were used in far- and near-UV spectral regions, respectively. The concentrations of the proteins were about 0.9 mg/ml. Each CD spectrum, in both far- and near-UV regions, represents the average of nine scans. CD spectra were recorded in mdegrees and then converted to mean residue ellipticity in $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$.

2.7 Steady-State Fluorescence spectroscopy

Steady-state fluorescence measurements were made with a Jobin Yvon-SPEX (Edison, NJ) Fluorolog tau-3 spectrofluorometer, the bandwidths for excitation and emission were 2 nm and 3 nm, respectively. The excitation λ of 295 nm was used to ensure that light was absorbed almost entirely by a tryptophanyl group. Protein solutions with about 0.07 OD at 295 nm were analyzed. All spectra were obtained from samples in 10 mM sodium phosphate at pH 7.3. The fluorescence spectra were corrected for light scattering from buffer and then for the instrument response by means of the appropriate correction curve. The quantum yields of the Trp residues in the proteins were calculated using a fluorescence standard, NATA. Quantum yield (Q) of NATA was taken as 0.13 [32]. To improve accuracy in calculations of the quantum yields as well as the overlap integrals (see below), the blue sides of the emission spectra were constructed using the log-normal function as described previously [28].

2.8 Fluorescence equilibrium binding assays

The solutions of xp-TL (about 4 μM) were titrated with DAUDA and the fluorescence spectra were measured. The excitation wavelength was 360 nm. The concentration of ethanol stock solution of DAUDA was confirmed by the absorbance at 335 nm, using an extinction coefficient, $\epsilon_{335} = 4800 \text{ M}^{-1} \text{ cm}^{-1}$. Following each addition of the ligand, the solution was mixed and allowed to equilibrate for 3 minutes. At the end of each titration experiment, the ethanol concentration did not exceed 2%. For proper deconvolution of the steady-state binding curves into the lifetime components, data in binding curves were taken as the integral intensity of DAUDA spectra after multiplication by the filter function (transmittance spectrum) of the Semrock filter (482/35). The binding data were analyzed with the following formula for one binding site, derived from the law of mass action:

$$y = 0.5 \times F \times \left[\left(1 + \frac{K_d}{n \times P} + \frac{L_t}{n \times P} \right) - \sqrt{\left(1 + \frac{K_d}{n \times P} + \frac{L_t}{n \times P} \right)^2 - \frac{4L_t}{n \times P}} \right]$$

where F is a fluorescence scaling factor, K_d is the apparent dissociation constant, P is the total protein concentration, L_t is the total ligand concentration, and n is the stoichiometry. In this case, where the ligand binding to TL occurs with 1:1 ratio, n represents a “correction” factor for the protein concentration. The deviations from the value 1 were within 5%. Correction is necessary to ensure the concentrations used are that of the functional monomeric proteins and that potential dimer fractions of the protein are negligible.

In the equilibrium competition binding assay, the TL-DAUDA complex (4 μ M and 3 μ M, respectively) was titrated with palmitic acid. After each addition of the competitor ligand, the solution was incubated about 3 minutes. The concentration of palmitic acid causing fluorescence decay to half-maximal intensity was taken as IC_{50} values. The apparent K_{diss}

value was calculated as in [33]: $K_{diss} = \frac{IC_{50}}{1 + [L]/K_d}$, where $[L]$ is the concentration of the fluorophore labeled lipid (DAUDA), and K_d is the protein-DAUDA complex dissociation constant.

Steady-state fluorescence measurements in the equilibrium competition binding assay were the same as described above for DAUDA.

2.9 Time dependent binding measurements by steady-state fluorescence

Time dependent binding studies were performed with NBDC₁₂-HPC (4 μ M) and NBD cholesterol (2 μ M) in 10mM sodium phosphate buffer at pH 7.3. The proteins, final concentrations, 0.2 μ M (TL or mutant S61/S153), were added to the ligand solutions while constantly stirred. The ligand binding kinetics were performed at least 3 times for each experiment. For both NBD-labeled ligands, 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 binding curves were analyzed with a single exponential function, $y = y_0 + A(1 - \exp(-t/\tau))$, where y_0 , 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.

2.10 Time-resolved fluorescence measurements

Time-resolved intensity decay data were obtained using a HORIBA Jobin Yvon MF² phase/modulation multi-frequency domain fluorometer. For Trp fluorescence, the excitation wavelength was 295 nm (LED). Emission was detected through a combination of filters (Semrock LP02-325RS-25 and Corning 7–51) that provide a bandpass of 325–410 nm. The available frequency range for the modulation of the excitation light intensity was 0.1–310 MHz. Phase angle and modulation of emission were measured by cross-correlation detection as described [34]. P-terphenyl in ethanol was used as a reference standard ($\tau = 1.05$ ns). For DAUDA fluorescence, the excitation wavelength was 360 nm (LED). The emission was detected through a Semrock filter (482/35). POPOP (1,4-bis(5-phenyloxazol-2-yl)benzene) in ethanol was used as a reference standard for DAUDA ($\tau = 1.32$ ns). Data analyses were performed with nonlinear least-squares programs from the Center for Fluorescence Spectroscopy (M. L. Johnson), University of Maryland at Baltimore, School of Medicine (Baltimore, MD). The goodness of fit was assessed by the χ^2 criterion.

The intensity decay data were analyzed in terms of the multiexponential decay law:

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i)$$

where α_i and τ_i are the normalized preexponential factors and decay times, respectively. The fractional fluorescence intensity of each component is defined as $f_i = \alpha_i \tau_i / \sum \alpha_j \tau_j$. Mean lifetime (intensity averaged) was calculated as $\tau_{av} = \sum f_i \tau_i$. However, the amplitude averaged lifetime, $\langle \tau \rangle = \sum \alpha_i \tau_i$, was used for the calculation of efficiency of FRET [35, 36]. The radiative rate constant was calculated as $k_r = Q / \langle \tau \rangle$. The fluorescence lifetime data represent the average of at least 3 independent experiments, each of which is the average of 5 to 7 measurements.

In DAUDA binding experiments, the fluorescence intensity decay data were analyzed simultaneously where lifetimes were considered as global parameters, i.e., the lifetimes are the same in each data set. The goodness of the model was judged by the global χ^2 criterion. Three lifetimes were necessary for acceptable fitting in the global fluorescence decay analysis. The fractional fluorescence intensity (f_i) was used for deconvolution of binding curves. The relative contribution of each lifetime component to the steady-state fluorescence intensity (I_{SS}) was calculated as $I_i = f_i I_{SS}$.

2.11 Solvent accessibility of the Trp17 by acrylamide quenching of fluorescence

The fluorescence of S61/S153 and TL were quenched by the progressive addition of various amounts of small aliquots of 8.0 M acrylamide solution. To ensure minimal contribution from Raman scatter, the fluorescence intensity was monitored at $\lambda = 338$ nm ($\lambda_{ex} = 295$ nm). Corrections for dilution of the sample and for inner filter effects produced by acrylamide absorption were performed as previously described [37]. The quenching data were fit to the Stern-Volmer formula, $(F_0/F) = 1 + (K_{SV}[Q])$, where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively. $[Q]$ is the concentration of the quencher and K_{SV} is the dynamic quenching constant ($K_{SV} = k_q \tau_{av}^0$). k_q is the bimolecular collisional rate constant and τ_{av}^0 is the intensity averaged fluorescence lifetime in the absence of the quencher.

2.12 Distance measurements by FRET

Resonance energy transfer from the Trp residue (donor) to the labeled 1,5 ANS group (acceptor) gauged the distance between the residues 62 and 81 (Trp62-1,5ANS81) in different conditions. The efficiency of FRET, E , was calculated from

$$E = 1 - (\langle \tau_{DA} \rangle / \langle \tau_D \rangle); E_{corr} = E / f_a$$

where $\langle \tau_{DA} \rangle$ and $\langle \tau_D \rangle$ are the fluorescence lifetimes of the donor in the presence and absence of acceptor, respectively. f_a is the fraction of dansyl labeling. E_{corr} is the efficiency of FRET corrected for incomplete labeling of the acceptor.

According to Förster's theory [38], the efficiency of FRET, E , between a donor, D, and an acceptor, A, is given by $E_{corr} = R_0^6 / (R_0^6 + R^6)$, where R is the distance between the donor and the acceptor. Estimation of the Förster distance, R_0 (in angstroms) at which 50% energy transfer occurs, has been described previously [28].

2.13 Urea equilibrium unfolding

In equilibrium unfolding measurements, TL and mutant proteins, 0.9 mg/ml, were incubated with various concentrations of urea 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 $Y = (\theta_N - \theta)/(\theta_N - \theta_D)$. 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 [39] by the nonlinear least-squares method using OriginPro version 8 (OriginLab Corp., Northampton, MA).

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

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.

Urea equilibrium unfolding of the mutant S61/S153 was also studied by fluorescence spectroscopy. The mutant protein was incubated with various concentration of urea for at least 18 hours. The fluorescence spectra were measured with excitation λ of 295 nm on solutions with absorbance of about 0.04. For each spectrum corrected for appropriate buffer the fluorescence spectral center of mass (SCM) was calculated using the following formula:

$$SCM = \frac{\sum I(\lambda_i) \lambda_i}{\sum I(\lambda_i)}$$

where $I(\lambda_i)$ is the fluorescence intensity at wavelength λ_i that was sampled in 0.5 nm intervals. To increase accuracy of SCM data the blue side of each spectrum (280–310 nm) was constructed using the log-normal function [28]. Fluorescence denaturation data were analyzed using the formula described above for CD data.

3. Results and discussion

The most significant result of this study is the elucidation of the mechanism of disulfide induced alteration of ligand binding in TL. Toward understanding this mechanism the topographical relationship of the disulfide bond and ligand positions are depicted (Figure 1) in the crystal structure for the apo- and holo-forms of TL. 1,4 butanediol, the artificial ligand in the crystal structure [20], is smaller than native ligands of TL. Despite the fact that the ligand is located deep in the cavity, the superficially positioned loop AB and the hairpin CD are most affected by the ligand binding. The native ligand, palmitic acid, shown as adapted from solution structure data [19] is poised to impact these regions even more than 1,4 butanediol.

3.1 Advantage of the use of xp-TL over apo-TL (delipidated) for mechanistic study

Previously, it has been shown that xp-TL and mutants contain some fatty acids [15, 40]. However, for ligand binding kinetics as well as FRET experiments, use of xp-TL without delipidation offer several advantages over applying apo-TL. In order to approach complete removal of ligands from TL, the delipidation procedure with the mixture of chloroform/

methanol should be performed at least three times [41]. Native-TL as well as xp-TL is a monomer [41]. The delipidation procedure, which denatures the protein, induces the oligomerization of TL. Dimer and higher-oligomers formations (up to 25%) have been shown in apo-TL [41]. Unfortunately, dimeric TL is not in equilibrium with the monomeric form. Furthermore, delipidation of TL modifies the near-UV CD intensity of the protein and manifests conformational changes. Saturation of apo-TL with stearic acid only partially restores the near-UV CD spectrum [42]. Published data indicate that oligomeric fractions, induced by delipidation procedure, are not functional in lipid binding. Direct calculation of the fractions of bound and free fatty acids that bind to apo-TL were determined by electron paramagnetic resonance. Stoichiometric ratios of fatty acid to apo-TL were as low as 0.65 suggesting that a portion of the protein is not functional [1]. Oligomer formation observed in the delipidation procedure may lead to significant distortion of FRET experiments, because about 25 % of the protein is not functional. The rate of FRET is proportional to R^{-6} , the donor-acceptor distance [36]. Therefore, a mixture with oligomer fractions of apo-TL may overshadow results from the monomer (the functional part of the protein).

Previously published results indicate that xp-TL can successfully be used as “apo”-protein to study the ligand induced changes in the protein [19, 21]. Xp-TL, complexed with the fatty acid analog, C12SL, or palmitic acid, displays distinct structural attributes [19]. Position specific complex formations with both ligands were consistent with structural features of TL. Furthermore, pH dependent conformational changes have been examined for xp-TL and PA-TL [21]. From many tested mutants, only mutant W28 has displayed a significant difference in pH titration curves for xp- and PA-TL. The conclusion, based on these results, is that the protonation states of the residues around position 28 modulate conformational switches of the AB loop relevant to ligand binding [21]. This conclusion was verified independently by crystallography of holo-TL. Specifically, the protonation switch resides at Glu27 [21]. Finally, xp-TL, without delipidation, has been used in ligand binding studies for comparative analysis of ten human lipocalins [43]. The dissociation constants for DAUDA, retinol, retinoic acid binding with xp-TL closely match our previous findings with delipidated-TL [15, 44]. All above-mentioned data clearly demonstrate that use of xp-TL as “apo-protein” is preferable to that treated excessively with methanol/chloroform mixture for more complete delipidation.

3.2 Molar extinction coefficient of TL

The molar extinction coefficient of TL determined by the Gill and von Hippel method [45] using 6M Gdn HCl as a denaturant was $13,000 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm. This number precisely matches the previously reported value [46]. However, the molar extinction coefficient of TL using 10M urea as the denaturant [47] yielded the value of $13,730 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm. The biuret method [30] applied over 20 samples of TL expressed at various times gave the average value of $13,790 \text{ M}^{-1}\text{cm}^{-1}$, which closely matches that determined with 10 M Urea. Therefore, the average extinction coefficient of $13,760 \text{ M}^{-1}\text{cm}^{-1}$ was used for TL in this study.

3.3 Structural integrity and stability of TL upon disulfide removal

The far-UV CD spectra of xp-TL and its mutants are almost identical (Figure 2). The mutations that disrupt the disulfide bond do not alter the secondary structure of TL. However, compared with xp-TL, the near-UV CD signal intensities of the mutants are significantly diminished. The shapes of the difference CD spectra are very similar to that of xp-TL (Figure 2B, upper inset). Disulfide bond removal results in decreased asymmetry of aromatic acid chains in all these proteins. Taken together it is reasonable to conclude that, removal of the conserved disulfide bond does not affect the secondary structure of TL, but triggers conformational relaxation. Previously, the removal of the disulfide bond with the

chemical reagent, β -mercaptoethanol, produced similar results.[22] The concordant results from removal of the disulfide bond by either chemical reduction or cysteine substitution indicate that the nature of the relaxation of the protein in both cases is the same. Conformational relaxation observed in the mutant proteins is not induced by the mutation *per se*, but rather by the disruption of the disulfide bond. However, there are some variations in the CD intensities between the mutant proteins where one or both Cys residues (61 and 153) are substituted. Inspection of the baseline differences in the CD spectra of the mutant proteins (Figure 2B) provides a partial explanation. Ratios of the CD intensities between 260–265 nm and 315–320 nm (starting points of the baselines) are different between the mutant proteins (Figure 2B). Therefore, baseline discrepancies appear more pronounced in the difference CD spectra (Figure 2B, upper inset). In fact normalization of the intensities for the CD spectra of the mutants S61, S153 and S61/S153 results in superimposition of the critical 270–295 nm region (Figure 2B, lower inset). Therefore, the nature of the relaxation that decreases the aromatic side chain asymmetry is essentially the same for all three mutants.

The intensity and sign (positive or negative) of the near-UV CD spectra of proteins depend on the spatial arrangement of their aromatic amino acid residues as well as the nearest neighboring residues within 10 Å [48]. TL contains one Trp, five Tyr and three Phe residues. The near-UV CD spectrum of TL reflects essentially the spectral features of Trp and Tyr residues [15]. Trp and Tyr side chains in TL derive their CD intensities from interactions with another aromatic side chains (His, Tyr, Trp, Phe) that are located within 10 Å [48]. The wavelength positions of the vibrational bands of the CD spectra of Trp and Tyr depend on their nearest environment (polarity, nearby charged group, etc.) [49, 50]. Therefore, the features of the near-UV CD spectra of proteins reflect specific tertiary structure and are unique for each protein.

The shapes of the spectra of TL and the mutant proteins are very similar to each other (Figure 2B). The relative spatial arrangement of the aromatic residues is therefore maintained. The reduced signal intensities in mutants S61, S153 and S61/S153 imply a gain in conformational freedom, relaxation.

The CD intensities from the conformational substates of aromatic amino acid residues located in the loop region vary in sign and intensity. Therefore, the CD signals of the same residue from this region cancel each other and do not make a significant net contribution. Consequently, rearrangements in these regions that may occur by the removal of disulfide bond are unlikely to directly influence the CD spectra. Yet, the loop rearrangements may change solvent accessibilities of the side chains of the residues located in the cavity.

To further characterize the nature of the structural relaxation induced by removal of the disulfide bond, fluorescence properties of the conserved Trp17 in TL were compared with that in S61/S153. The steady-state fluorescence spectra of Trp17 in both proteins are shown in Figure 3A. The fluorescence λ_{max} values of Trp17 in both proteins are almost identical. Therefore, removal of the disulfide bond in TL does not alter the polarity or the charge distribution around the side chain of Trp17. However, the quantum yield of Trp17 in S61/S153 (0.038) is increased about 2.4 fold compared with that obtained in TL (0.016) (Figure 3A). Thus, the fluorescence of Trp17 in S61/S153 is relatively unquenched. The two mechanisms of quenching, dynamic and static, can be distinguished by fluorescence lifetime measurements. Static quenching arises from the non-fluorescent ground state complex formation between the fluorophore (Trp) and “quencher” groups. The “quencher” groups for Trp fluorescence could be the amino acid side chains, which may act as electron acceptors or proton donors [51]. The excited states of these complexes are quenched before the fluorescence event and do not participate in the fluorescence decay. As a result, fluorescence

lifetimes are not affected by static quenching. Because non-fluorescent complexes absorb photons, steady-state fluorescence intensity and quantum yield are affected by static quenching. On the other hand, dynamic quenching originates from the quenching process that occurs during the lifetime of the excited state. Therefore, both steady-state fluorescence intensity and fluorescence lifetime are affected by dynamic quenching.

To characterize the quenching mechanism, fluorescence lifetime measurements were performed. Frequency-domain fluorescence intensity decays for Trp17 in TL and S61/S153 are shown in Figure 3B. Fluorescence lifetime parameters obtained from the double-exponential decay model are shown in Table 1. The amplitude-averaged lifetime $\langle\tau\rangle$, which is proportional to the steady-state intensity, of Trp17 in S61/S153 (1.08 ns) is increased about 2.5 fold compared with that of TL (0.44). Removal of the disulfide bond results in the essentially same fold increase in quantum yield and amplitude-averaged lifetime. Therefore, the unquenching of the fluorescence that occurs in S61/S153 versus TL is dynamic. As expected in the absence of static quenching, the radiative rate constants, k_r , for TL and S61/S153 are almost the same, about $36 \times 10^6 \text{ s}^{-1}$ (Table 1). This value is close to the k_r data obtained for 3-methylindole, tryptophan zwitterions, and N-acetyltryptophanamide ($(40\text{--}60) \times 10^6 \text{ s}^{-1}$) [52]. Consequently, exiguous static quenching can be expected for Trp17 in both TL and S61/S153. Thus, lifetime fluorescence parameters of Trp 17 in TL and S61/S153 are consistent with the notion that removal of the disulfide bond induces relaxation of the protein. Steady-state parameters exclude denaturation. Denaturation of the protein should result in a red shift of the fluorescence λ_{max} that is not observed for the S61/S153 (Figure 3A). The impact of the removal of the disulfide bond on solvent accessibility was further investigated in acrylamide quenching of Trp in TL and S61/S153. The side chain of Trp17 is buried at the closed end the barrel [1, 2] (Figure 3A, inset). Significant structural changes in TL should change the solvent accessibility of the side chain of Trp17. The Stern-Volmer plots for Trp17 in TL and S61/S153 are shown in Figure 4. The bimolecular collisional rate constant, k_q , values, which are attributable to the accessibility, are basically same for TL and S61/S153, about $0.8 \text{ M}^{-1}\text{ns}^{-1}$. Thus, the removal of the disulfide bond does not change the solvent accessibility to the side chain of Trp17. These data are consonant with the absence of a denaturation event in the protein S61/S153. The quenching constant, K_{SV} , for Trp 17 in S61/S153 is increased about 2 fold, from 1.02 to 2.15 M^{-1} , compared with that in TL. However, K_{SV} value depends on fluorescence lifetime and can not be used as the measure of accessibility [37].

Urea equilibrium unfolding experiments were performed to assess the stability of TL with and without the disulfide bond. In agreement with the near-UV CD results, the absence of the disulfide bond in TL results in unfolding at a much lower concentration of urea than with the intact disulfide (Figure 5 and Table 2). The disruption of the disulfide bond decreases the free energy of unfolding from 4.9 ± 0.2 to $2.1 \pm 0.3 \text{ kcal/mol}$. A two-state model (Materials and methods) is sufficient to describe the unfolding of both the disulfide disrupted mutants and the disulfide intact protein. Use of a three-state model does not significantly improve the fitting of the unfolding data (data not shown). For proteins with a two-state unfolding mechanism, the amount of surface area accessible to solvent upon unfolding is a main determinant for the m value [53]. Often the disulfide reduction of proteins results in increased m values [54]. Despite the significant decrease in stability, or m values, the dependence of the free energy of unfolding on denaturant concentration are not significantly changed (Table 2). The similar m values observed for the mutant proteins and TL suggest that difference in the surface area accessible to solvent upon denaturation is minimally altered after the removal of the disulfide bond.

Urea unfolding studies were previously performed for PA- and xp-A101 mutants of TL using both near- and far-UV CD data [40]. Unfolding curves derived from near-UV- and far-

UV CD experiments were superimposable; secondary and tertiary structure change in unison. The two-state model was satisfactory to describe unfolding of the A101 mutant of TL [40]. However, one could argue that the removal of the disulfide bond may change unfolding mechanism of the protein. Therefore verifying the pattern of unfolding by another method is advantageous. Accordingly, urea unfolding of S61/S153 was also performed with steady-state fluorescence of Trp17 (Figure 6). As expected, fluorescence $\lambda_{\max}$ of Trp 17 displays the progressive red shift with unfolding in urea. To reveal possible stable intermediate states the SCM values, not fluorescence $\lambda_{\max}$, are preferred (Figure 5). The unfolding curve of S61/S153 by fluorescence is similar to that of the far-UV CD (Figure 5). The recovered ΔG^0 value for S61/S153 from fluorescence experiment (2.4 ± 0.2) is very similar that obtained from far-UV CD experiment (2.1 ± 0.3). However, the m value from the fluorescence measurements is somewhat higher.

Comparison of these findings with the previous results where the disulfide bond has been reduced by the chemical reagent is revealing. Although only one concentration of urea (8 M) has been used to test the stability of TL by far-UV CD [22], the results shown in Figure 5 corroborate previous findings. Complete unfolding of the protein at 8M urea was observed only after the reduction of the disulfide bond with β -mercaptoethanol [22]. All mutants without the disulfide bond are completely unfolded at 8.0 M urea, while residual structure is evident for xp-TL (Figure 5). The results substantiate that the effect observed from the mutations is limited to the disulfide bond removal.

3.4 Characterization of the fatty acid binding site of TL with and without the disulfide bond

A fluorescent labeled fatty acid, DAUDA, was used to characterize the binding site of TL. DAUDA bound to TL displays an enhancement of the fluorescence intensity and a blue shift in the fluorescence $\lambda_{\max}$ [15]. A blue shift in the fluorescence of DAUDA depends on the polarity of the environment [55]. The fluorescence spectra of DAUDA bound to xp-TL and xp-S61/S153 are almost superimposable. For both, fluorescence $\lambda_{\max}$ values are at about 500 nm, which indicate a hydrophobic environment (Figure 7A). The extent of the spectral blue shift is evident in the normalized fluorescence spectrum of free DAUDA, $\lambda_{\max} \sim 554$ nm, (Figure 7B). The results show that the polarity of the fatty acid binding site of TL is not altered by removal of the disulfide bond.

The steady-state fluorescence of bound DAUDA may originate from various species that display distinct lifetimes. Therefore, steady-state and time-resolved fluorescence methods were applied in tandem to ligand binding to characterize the individual components. The deconvolutions of the binding curves into the lifetime components, generated from the global decay time analysis (Table 3 and 4), are revealing (Figure 7B and 7C). In both proteins, xp-TL and xp-S61/S163, the major contributions to steady-state intensities come from the long lifetime species, which have a fluorescence lifetime of about 22.5 ns. For DAUDA binding, dissociation constants for the long lifetime components match for xp-TL and xp-S61/S153, 2.4 ± 0.3 and 2.6 ± 0.3 μ M, respectively, (Figure 7B and 7C). In accord with steady-state data, the fatty acid binding site of TL is not modified by the disruption of the disulfide bond. The contributions of fluorescence lifetime components 0.50–0.58 ns to the steady-state fluorescence do not exceed 3%, and are most likely associated with scatter. Free DAUDA in the same buffer exhibits the lifetime of about 3.8 ns with a small contribution from 0.40 ns (data not shown). The dissociation constants for the species that have fluorescence lifetimes of about 5 ns are also similar (Figure 7B and 7C). A ten fold increase in dissociation constants (about 25 μ M) can be related to non-specific binding. Consistent with this description, the lifetime of 5 ns is close to that of free DAUDA, and indicates that these species are highly solvated. In DAUDA titration experiments, the component (3.8 ns) corresponding to the free DAUDA is not resolved and apparently mixed

with the component of 5 ns. Therefore, the dissociation constants for the component of ~5 ns are less accurate.

To validate the assignment of the binding species, the steady-state and time-resolved fluorescence were applied to the competitive displacement experiment with palmitic acid. Palmitic acid binds in the cavity of TL and displaces the bound DAUDA from a hydrophobic environment to the solvent [15]. The displacement results in diminished fluorescence intensity and a red shift in the fluorescence λ_{max} (Figures 8A). Deconvolution of the displacement curve into the lifetime components indicates that the species with ~22 ns fluorescence lifetimes are associated with the fatty acid binding site. The lifetime of the shorter lifetime component, about 3.6 ns, is closer to that of free DAUDA (Figure 8B and Table 5).

3.5 Kinetics of ligand binding for TL with and without the disulfide bond

The results with DAUDA experiments are somewhat surprising. Previous experiments have shown that reduction of the disulfide bond significantly increases binding affinities of retinol and retinoic acid to TL, along with structural relaxation [22, 23]. Because the disulfide bond reduction induces structural relaxation in TL, we hypothesized that ligand size or bulkiness could be the factor responsible for the different outcomes. To test this concept and, in addition, to reveal dynamic information, two dissimilar yet bulky ligands, NBD C₁₂-HPC and NBD cholesterol, were selected for the time-dependent binding with xp-TL and xp-S61/S153 (Figure 9). Clearly, TL without the disulfide bond discriminates the ligands. The time constant for the NBD cholesterol binding to xp-S61/S153 is decreased about three fold (3.3 ± 0.1 from 11.8 ± 0.2 s) compared with that of xp-TL. However, only a modest decrease, about 30 %, is observed in the experiment with NBD C₁₂-HPC. The time constants for NBD C₁₂-HPC binding to xp-TL and S61/S153 are 32.2 ± 0.2 and 22.2 ± 0.4 s, respectively. The results are consistent with enhanced plasticity for TL with the disulfide bond deleted. S61/S153 adopts the ligands faster than does TL, but the ligand type is the factor mediating the activity.

3.6 FRET between the loops CD and EF to elucidate mechanism of the ligand binding conferred by disulfide bond removal

The conserved disulfide bond links the hairpin CD to the C-terminus of the protein. Despite this conformational restriction, the structural comparison of the crystal structures of apo- and holo-TL has demonstrated significant conformational changes in the hairpin CD (Figure 1) [2, 20]. Therefore, it is reasonable to expect that disruption of the disulfide bond will directly influence the dynamics of the hairpin CD. To reveal the conformational changes in the ligand-TL with and without the disulfide bond, FRET was applied to measure distance between residues 62 and 81 in various states using the construct Trp62-1,5ANS81. Fluorescence lifetime parameters of the two sets of the mutants W62C81 (Trp alone and Trp-1,5ANS pair) at various states are shown in Table 6. In all cases, three lifetimes are sufficient to describe the fluorescence intensity decays. As an example, frequency-domain intensity decay curves for the mutant W62C81, with and without the 1,5ANS label, in disulfide reduced and oxidized forms are shown in Figure 10. The data for the 1,5-ANS labeled proteins compared with their unlabeled counterparts are shifted toward the higher frequencies indicating relatively lower average lifetimes. In Figure 10, the extent of the horizontal shift between the same phase angle values is a good practical guide for the magnitude of the lifetime decrease and, therefore, the efficiency of FRET. The parameters for FRET and measured distances are shown in Table 7. For comparison, the corresponding distances from the crystal structures of apo- and holo-TL are also presented where it is relevant. The distance changes in the crystal and solution structures follow the same tendencies (Table 7). In both disulfide reduced and intact forms, the distance between the

loops CD and EF is decreased in PA-TL compared with that of xp-TL. This distance is further decreased by the reduction of the disulfide bond. Thus, removal of the disulfide bond stimulates flexibility of the protein to reach an inter-loop distance, which is not accessible for the protein with the intact disulfide bond. Accordingly, enhanced flexibility of the protein promotes a faster accommodation of the ligand inside the cavity and energetically more favorable ligand-protein complex. The discrepancies in the distance values between the two methods reflect the fact that distances from the crystal structure are taken using C α atoms of the residues. However, FRET measurement assigns the center-to-center distance between the indole group of the Trp62 and the 1,5-ANS group at position 81. Therefore, different distance values obtained by two methods can be rationalized as the consequence of the projection of the Trp side chain and/or the probe arm away from the backbone of TL. It should be noted that all experiments were performed with the same stock solution of the labeled protein (Trp62-1,5ANS81). Uncertainty in the labeling efficiency is the same in all cases. Therefore, relative variations of the distances observed in different states of the protein are quite accurate.

3.7 Effect of removal of the conserved disulfide bond in the lipocalin family

Effect of the conserved disulfide bond removal on structure and function has been studied for the other members of the lipocalin family. The findings for equine β -lactoglobulin are similar to that of TL. Removal of the conserved disulfide bond by mutation (C66A/C160A) did not change secondary structure but decreased stability against urea denaturation [56]. However, the disulfide bond decreased the free energy of unfolding of equine β -lactoglobulin about 1.6 kcal/mol, which is significantly less than that obtained for TL (2.8 kcal/mol). In another lipocalin, rat lipocalin-type prostaglandin D (L-PGDS), deletion of the conserved disulfide bond via mutation (C186A or C89A/C186A) did not alter the far-UV CD, hence, secondary structure of the protein [57]. However, disulfide bond removal significantly reduced the stability of the protein against urea as well as thermal unfolding. Similar to TL, but to a much lesser extent, disulfide bond removal is associated with increased ligand affinity of the protein. In contrast, porcine odorant binding protein (p-OBP) showed a six fold decrease in binding affinity to the ligand, in spite of intact secondary structure [58]; however, removal of the disulfide bond decreased stability of the protein.

The enzymatic reduction with the thioredoxin system has also been shown with bovine β -lactoglobulin. The tertiary structure closely resembles that of tear lipocalin [59]. The reduced form of β -lactoglobulin is very sensitive to pepsin digestion. Allergenicity is lost after the enzymatic reduction of one or both of its disulfide bonds.

All the above-mentioned data suggest that the conserved disulfide bond that links the hairpin CD to the C-terminus may be functionally relevant to all members of the lipocalin family. Common to all lipocalins that have been studied is the reduction of protein stability to varying degrees in the absence of secondary structure changes with removal of the disulfide. However, deletion of the disulfide bond alters the binding function of the lipocalins in both ligand- and protein-specific manners.

3.7 Possible functional implications of disulfide mediated ligand binding

TL is a multifunctional protein. Although TL is the principal lipid binding protein in human tears [12], it is also found in other human tissues [60–63]. The dominant function of TL may vary significantly from one tissue to another. The common feature in all functions of TL is the ability to bind various classes of ligands. Disulfide bonds may act as dynamic scaffolds to present proteins in different conformational and functional states [64]. As this concept has been recently extended to only a few extracellular proteins, it is intriguing to consider the highly conserved disulfide bond of the lipocalin family in this role [65]. The selective

response to ligand binding upon thiol-disulfide exchange in TL may have functional relevance. Both TL and thioredoxin are expressed in human prostate [23]. Interestingly, human thioredoxin interacts with TL, reduces its disulfide bond and enhances retinoic acid binding affinity about nine-fold. In the human tear film, the relevance of a mechanism for alteration of lipid affinity may be linked to the presence of thioredoxin. The concentration of thioredoxin was reported to markedly increase in human tears in subjects with inflammation [66]. A speculative example of where such a response would have functional impact is the anti-fungal activity of TL derived from siderophore binding. For the disulfide intact form of TL the association constant of rhodotorulic acid is slightly less than that of the naturally abundant stearic acid [5]. Rhodotorulic acid, like many fungal siderophores, are large cyclic compounds. Conformational relaxation of TL mediated by thioredoxin in response to inflammation could trigger a ligand swap, siderophore for fatty acid and boost anti-fungal activity.

Because of TL's selective response to disulfide bridge disruption, the possibility for a redox mediated substrate switch and delivery is plausible. This motif has implications for the conserved disulfide bond that is nearly ubiquitous in the lipocalin family.

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Abbreviations

CD	circular dichroism
DAUDA	11-((5-dimethylaminonaphthalene-1-sulfonyl)amino)undecanoic acid
FRET	fluorescence resonance energy transfer
1,5-IAEDANS	5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid
NATA	N-acetyl-L-tryptophanamide
NBD cholesterol	22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3-ol
NBD C₁₂-HPC	2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl- <i>sn</i> -glycero-3-phosphocholine
SDTF	site directed tryptophan fluorescence
TCEP	tris(2-Carboxyethyl) phosphine hydrochloride
TL	human tear lipocalin

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Figure 1.

Ribbon diagrams of the crystal structures of TL in apo- (PDB: 1XKI, cyan) and holo-forms (PDB: 3EYC, grey). For the FRET measurements, residues 81 and 62 were substituted with Cys (labeled with 1,5-ANS) and Trp, respectively. Large-sized balls show locations of the C_{α} atoms. The measured distances in apo- and holo-forms are indicated with double-headed arrows. Single capital letters show the identities of the β -strands. Double capital letters denote the identities of the loops at the open end of the ligand binding barrel. In holo-TL, the bound artificial ligand 1,4-butanediol is shown in CPK representation. Schematically, multiple positions of the palmitic acid (stick and semi-transparent surface representation) in the cavity of TL show energetically favored positions in accord with [19]. In holo-TL, the disulfide bond between the residues Cys61 and Cys153 is shown in stick representation. In apo-TL, only Cys61 of the disulfide bond is shown because C-terminal end with Cys153 was not resolved in crystal structure. DS Visualizer 2.5 (Accelrys Inc.) was used to generate the ribbon diagrams of the proteins.

Figure 2.

CD spectra of xp-TL and the xp-TL mutants. In each xp-TL mutant, amino acid substitution eliminated the conserved disulfide bond. (A) Far-UV and (B) near-UV spectral regions. Symbols shown in (A) correspond to the plots for each protein in both (A) and (B). Buffer: 10 mM sodium phosphate, pH 7.3. (Inset, upper) Difference CD spectra (black: TL-S61/S153; gray: TL-S61; light gray: TL-S153). (Inset, lower) intensity normalized near-UV CD spectra of the mutants. Symbols are the same as in (B).

Figure 3.

Fluorescence properties of the conserved Trp17 in TL and S61/S153. (A) Steady-state fluorescence spectra of Trp17 in TL (solid line) and S61/S153 (dashed line). The absorbance at 295 nm for the solution of both proteins is 0.045. A, inset, depict the location of the side chain of Trp17 (stick representation) in the protein scaffold (line ribbon representation). (B) Phase angle (circles) and modulation (triangles) data for the fluorescence intensity decays of Trp17 in TL (solid symbols) and S61/S153 (open symbols). Lines represent the best double-exponential fit for the parameters given in the Table 1.

Figure 4. Stern-Volmer plot for acrylamide quenching of the fluorescence for Trp17 in TL and S61/S153.

Figure 5.

Urea denaturation curves for the xp-TL and the xp-TL mutants. (○) symbols denote the average values of the fractional changes for the mutants xp-S61, xp-153 and xp-S61/S153. The individual data for the mutant proteins (● S61/S153; $\Delta G^0 = 2.1 \pm 0.7$ kcal/mol, $m = 0.6 \pm 0.1$; ▲ S153; $\Delta G^0 = 2.5 \pm 0.6$ kcal/mol, $m = 0.7 \pm 0.1$; ○ S61; $\Delta G^0 = 2.3 \pm 0.5$ kcal/mol, $m = 0.6 \pm 0.1$) are nearly superimposable and shown in inset. (□) symbols denote the fractional changes for the xp-TL. Half-filled circle symbols denote the fluorescence spectral center of mass (SCM) values calculated from the fluorescence spectra of the S61/S152 (Figure 6).

Figure 6.

The fluorescence spectra of Trp17 in the S61/S153 at various concentrations of urea. To accentuate the spectral transition between folded and unfolded states, the spectra are shown for wavelength interval 300–420 nm. However, for better accuracy, the fluorescence spectral center of mass (SCM) values (in Figure 5) were calculated from the spectral range of 280–500 nm (see Materials and Methods).

Figure 7.

DAUDA binding to the xp-TL with and without disulfide bond. (A) Comparison of spectra of DAUDA bound to the xp-TL (solid line) and xp-S61/S153 (dashed line). To emphasize the extent of the blue-shift of the fluorescence spectra of bound-DAUDA, the normalized fluorescence spectrum of free DAUDA (solid line with open circle) is also presented. The concentrations of the xp-proteins and DAUDA were 3.7 μM and 0.2 μM , respectively, to ensure that free-DAUDA does not contribute to the fluorescence spectra. Deconvolution of the titration curves of DAUDA fluorescence into lifetime components for xp-TL (B) and xp-S61/S153 (C). In both cases, the concentration of the protein was 3.7 μM . The lifetime components of binding curves were obtained by simultaneous fitting decay curves to a triple-decay function where the lifetimes were set as global parameters. Solid curves are generated by fitting of the experimental data to one binding site model. f_i and I_{ss} are the fractional fluorescence intensity of each component and the steady-state fluorescence intensity, respectively.

Figure 9.

Influence of the conserved disulfide bond to ligand binding kinetics. (A) Time dependent binding of NBD C_{12} -HPC to xp-TL (grey symbols) and xp-S61/S153 (black symbols). (B) Time dependent binding of NBD-cholesterol to xp-TL (grey symbols) and xp-S61/S153 (black symbols). The arrows point to the ordinate for each plot. The time constants (τ) for the binding kinetics are shown in the figure.

Figure 10.

Phase angle (circles) and modulation (triangles) data for the fluorescence intensity decays of TL mutant W62C81 with (solid symbols) and without (open symbols) 1,5-ANS labeled Cys81 for detection of FRET. (A) The labeled and unlabeled proteins in xp-forms with intact disulfide bond. (B) The labeled and unlabeled proteins in PA-forms (saturated with palmitic acid with ratio of 1:3) with reduced disulfide (with 20 fold excess of TCEP). Lines represent the best triple-exponential fit for the parameters given in the Table 6. The difference between the decay curves, dotted lines in (A) and (B) indicate positions of the frequencies corresponding to the phase angle of about 63 degree.

Table 1

Fluorescence lifetime parameters for Trp17 in the xp-TL and S61/S153 at pH 7.3.

Protein	a_1^a	a_2	τ_1^b (ns)	τ_2 (ns)	Q^c	τ_{av}^d (ns)	$\langle \tau \rangle^e$ (ns)	$k_f \times 10^{-6}$ (s ⁻¹)	χ^2
Xp-TL	0.96 (± 0.01)	0.04 (± 0.01)	0.32 (± 0.01)	3.40 (± 0.14)	0.016	1.27	0.44	36.4	1.2
Xp-S61/S153	0.79 (± 0.02)	0.21 (± 0.01)	0.41 (± 0.01)	3.59 (± 0.06)	0.038	2.63	1.08	35.2	1.2

^a normalized preexponential factor;

^b decay time;

^c quantum yield;

^d intensity averaged lifetime;

^e amplitude averaged lifetime,

^f radiative rate constant

Table 2

The thermodynamics parameters of the proteins derived from fits to the two-state model for urea denaturation

Protein	ΔG^0 (kcal mol ⁻¹)	m (kcal mol ⁻¹ M ⁻¹)
Xp-TL	4.9± 0.2	0.72± 0.03
Xp-S61/S153	2.1± 0.3	0.60± 0.04
Xp-S61/S153*	2.4± 0.2	0.82± 0.05

*
from fluorescence SCM

Table 3

The fluorescence lifetime parameters for xp-TL (3.7 μ M), titrated with DAUDA. Global parameters: $\tau_1 = 0.58 \pm 0.15$ ns; $\tau_2 = 4.99 \pm 0.35$ ns; $\tau_3 = 22.2 \pm 0.25$ ns; $\chi^2 = 0.6$

DAUDA(μ M)	a_1	a_2	a_3
0.4	0.80 ± 0.03	0.08 ± 0.02	0.12 ± 0.02
0.8	0.73 ± 0.04	0.12 ± 0.02	0.15 ± 0.03
1.2	0.63 ± 0.04	0.17 ± 0.02	0.20 ± 0.03
1.6	0.57 ± 0.05	0.19 ± 0.03	0.24 ± 0.03
2.4	0.49 ± 0.06	0.24 ± 0.03	0.28 ± 0.03
3.3	0.45 ± 0.06	0.26 ± 0.03	0.28 ± 0.03
4.1	0.44 ± 0.01	0.27 ± 0.04	0.29 ± 0.03
5.7	0.39 ± 0.07	0.31 ± 0.04	0.31 ± 0.03
7.3	0.40 ± 0.07	0.30 ± 0.04	0.29 ± 0.03
10.5	0.40 ± 0.07	0.32 ± 0.03	0.28 ± 0.03
13.7	0.34 ± 0.07	0.38 ± 0.05	0.28 ± 0.03
16.9	0.34 ± 0.07	0.40 ± 0.05	0.27 ± 0.03
20.0	0.36 ± 0.07	0.40 ± 0.05	0.24 ± 0.02

Table 4

The fluorescence lifetime parameters for the TL mutant S61/S153 (3.8 μM) titrated with DAUDA.
Global parameters: $\tau_1 = 0.50 \pm 0.03$ ns; $\tau_2 = 5.12 \pm 0.13$ ns; $\tau_3 = 22.86 \pm 0.09$ ns; $\chi^2 = 0.8$

DAUDA(μM)	α_1	α_2	α_3
0.4	0.83 ± 0.01	0.08 ± 0.00	0.090 ± 0.00
0.8	0.75 ± 0.01	0.11 ± 0.01	0.13 ± 0.01
1.2	0.70 ± 0.01	0.13 ± 0.01	0.17 ± 0.01
1.6	0.64 ± 0.01	0.16 ± 0.01	0.19 ± 0.01
2.4	0.62 ± 0.02	0.17 ± 0.01	0.22 ± 0.01
3.3	0.57 ± 0.02	0.20 ± 0.01	0.23 ± 0.01
4.1	0.71 ± 0.01	0.14 ± 0.01	0.16 ± 0.01
5.7	0.55 ± 0.02	0.22 ± 0.01	0.24 ± 0.009
7.3	0.47 ± 0.02	0.27 ± 0.01	0.27 ± 0.01
10.5	0.42 ± 0.02	0.30 ± 0.02	0.28 ± 0.01
13.7	0.36 ± 0.03	0.37 ± 0.02	0.28 ± 0.01
16.9	0.36 ± 0.03	0.38 ± 0.02	0.26 ± 0.01
20.0	0.37 ± 0.03	0.38 ± 0.02	0.24 ± 0.01

Table 5

The fluorescence lifetime parameters for TL-DAUDA^a complex titrated with palmitic acid (PA).

Global parameters: $\tau_1 = 0.22 \pm 0.07$ ns; $\tau_2 = 3.63 \pm 0.13$ ns; $\tau_3 = 22.02 \pm 0.09$ ns; $\chi^2 = 0.9$

PA, (μ M)	a_1	a_2	a_3
0	0.65 ± 0.07	0.25 ± 0.07	0.20 ± 0.04
0.8	0.60 ± 0.07	0.18 ± 0.03	0.22 ± 0.04
1.6	0.61 ± 0.07	0.17 ± 0.03	0.22 ± 0.04
2.4	0.66 ± 0.07	0.16 ± 0.03	0.18 ± 0.04
4.1	0.73 ± 0.06	0.14 ± 0.03	0.13 ± 0.03
5.7	0.72 ± 0.05	0.16 ± 0.03	0.12 ± 0.03
7.3	0.76 ± 0.05	0.14 ± 0.03	0.10 ± 0.02
10.5	0.79 ± 0.04	0.14 ± 0.03	0.07 ± 0.02
13.7	0.80 ± 0.04	0.14 ± 0.03	0.08 ± 0.02
20.2	0.81 ± 0.04	0.14 ± 0.02	0.05 ± 0.01

^a χ p-TL (4.0 μ M)+ DAUDA (3.0 μ M)

Table 6

Fluorescence lifetime parameters for the TL mutant W62C81 (with and without 1,5-ANS label) in various conditions at pH 7.3.

Trp Mutant	a_1^b	a_2	a_3	τ_1^c (ns)	τ_2 (ns)	τ_3 (ns)	τ_{av}^d (ns)	$\langle \tau \rangle^e$ (ns)	χ^2
Xp-W62C81	0.32 (-0.01) (+0.01)	0.45 (-0.04) (+0.04)	0.23 (-0.04) (+0.04)	0.33 (-0.06) (+0.05)	2.51 (-0.19) (+0.20)	5.30 (-0.24) (+0.33)	3.79	2.45	0.6
Xp-W62ANS ^a 81	0.32 (-0.03) (+0.03)	0.33 (-0.02) (+0.01)	0.35 (-0.03) (+0.02)	0.39 (-0.13) (+0.10)	1.63 (-0.23) (+0.29)	4.41 (-0.13) (+0.17)	3.50	2.20	0.6
Xp-W62C81, disulfide reduced	0.31 (-0.01) (+0.01)	0.42 (-0.04) (+0.05)	0.27 (-0.05) (+0.04)	0.33 (-0.07) (+0.07)	2.33 (-0.23) (+0.25)	4.91 (-0.22) (+0.31)	3.68	2.42	0.6
Xp-W62ANS81, disulfide reduced	0.39 (-0.02) (+0.03)	0.34 (-0.01) (+0.03)	0.27 (-0.06) (+0.04)	0.43 (-0.11) (+0.09)	1.94 (-0.33) (+0.40)	4.58 (-0.23) (+0.39)	3.41	2.08	0.6
f PA-W62C81	0.34 (-0.01) (+0.01)	0.42 (-0.02) (+0.02)	0.24 (-0.03) (+0.02)	0.41 (-0.04) (+0.04)	2.21 (-0.12) (+0.13)	4.77 (-0.13) (+0.15)	3.42	2.21	0.4
PA-W62ANS81	0.42 (-0.03) (+0.03)	0.34 (-0.01) (+0.02)	0.24 (-0.04) (+0.03)	0.39 (-0.09) (+0.07)	1.79 (-0.24) (+0.30)	4.47 (-0.20) (+0.31)	3.22	1.84	0.6
PA-W62C81, disulfide reduced	0.34 (-0.01) (+0.01)	0.45 (-0.03) (+0.03)	0.21 (-0.04) (+0.03)	0.45 (-0.04) (+0.04)	2.37 (-0.15) (+0.15)	4.99 (-0.23) (+0.28)	3.45	2.26	0.4
PA-W62ANS81, disulfide reduced	0.41 (-0.01) (+0.01)	0.37 (-0.01) (+0.01)	0.22 (-0.02) (+0.02)	0.31 (-0.07) (+0.06)	1.69 (-0.15) (+0.18)	4.47 (-0.16) (+0.21)	3.16	1.73	0.6

^a 1,5 ANS;

^b normalized preexponential factor;

^c decay time;

^d intensity averaged lifetime;

^e amplitude averaged lifetime;

^f saturated with palmitic acid (ratio 1:3)

Table 7

FRET parameters and distance data for Trp(62)-1,5ANS(81) pair in various conditions at pH 7.3.

Trp Mutant	$\langle \tau \rangle^b$ (ns)	E^c	d_{fa}	E_{corr}^e	Q^f	$g \cdot 10^{-13}$ $M^{-1}cm^{-1}(nm)^4$	R_0^h (Å)	R^i (Å)	$j R_{Ca-\alpha}$ (Å)
Xp-W62C81	2.45				0.10				
Xp-W62ANS ^a 81	2.20	0.10	0.83	0.12		5.98	21.2	29.5	22.6
Xp-W62C81, disulfide reduced	2.42				0.10				
Xp-W62ANS81, disulfide reduced	2.08	0.14	0.83	0.17		5.98	21.2	27.6	
j PA-W62C81	2.21				0.09				
PA-W62ANS81	1.84	0.17		0.20		5.93	20.8	26.2	21.7
PA-W62C81, disulfide reduced	2.26				0.09				
PA-W62ANS81, disulfide reduced	1.73	0.23	0.83	0.28		5.90	20.8	24.3	

^a -1,5-ANS;

^b amplitude averaged lifetime;

^c the efficiency of resonance energy transfer;

^d a fraction of 1,5-ANS labeling;

^e the efficiency of resonance energy transfer corrected for incomplete labeling;

^f quantum yield;

^g the overlap integral;

^h the Förster distance (distance at which 50% energy transfer occur);

ⁱ donor-acceptor distance;

^j saturated with palmitic acid (ratio 1:3);

^k $C_{\alpha}-C_{\alpha}$ distances apo- and holo-TL were determined from the PDB files 1XKI and 3EYC, respectively.