Differential Contribution of Tryptophans to the Folding and Stability of the Attachment Invasion Locus Transmembrane β-Barrel from *Yersinia pestis*

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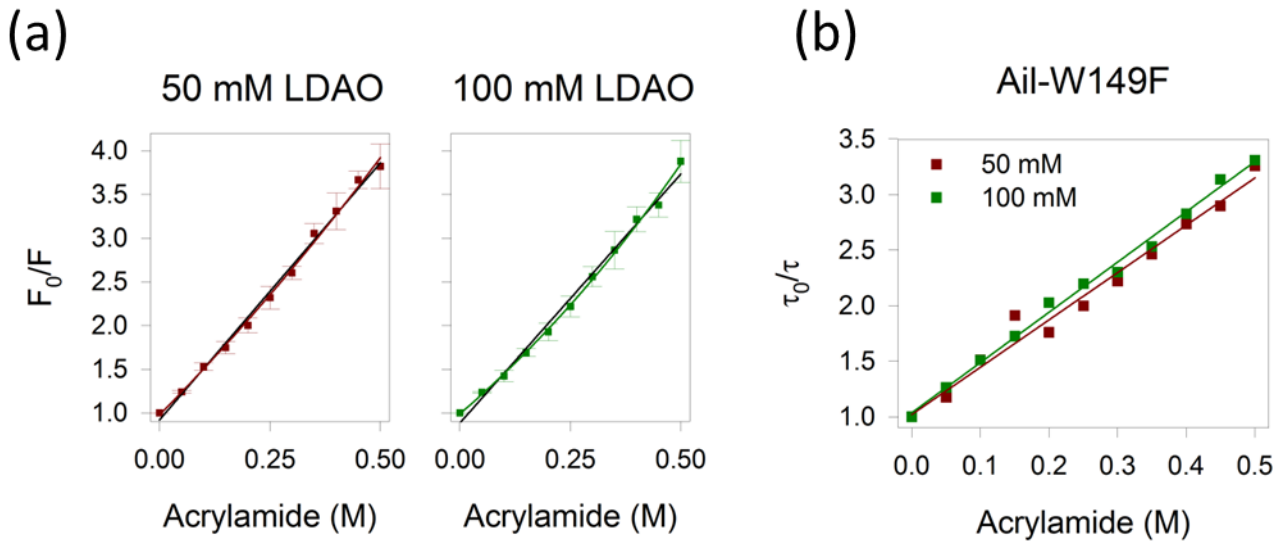
SUPPLEMENTARY INFORMATION

Supplementary Table 1. Apparent thermodynamic parameters derived for the folding and unfolding process of Ail compared with anisotropy data.

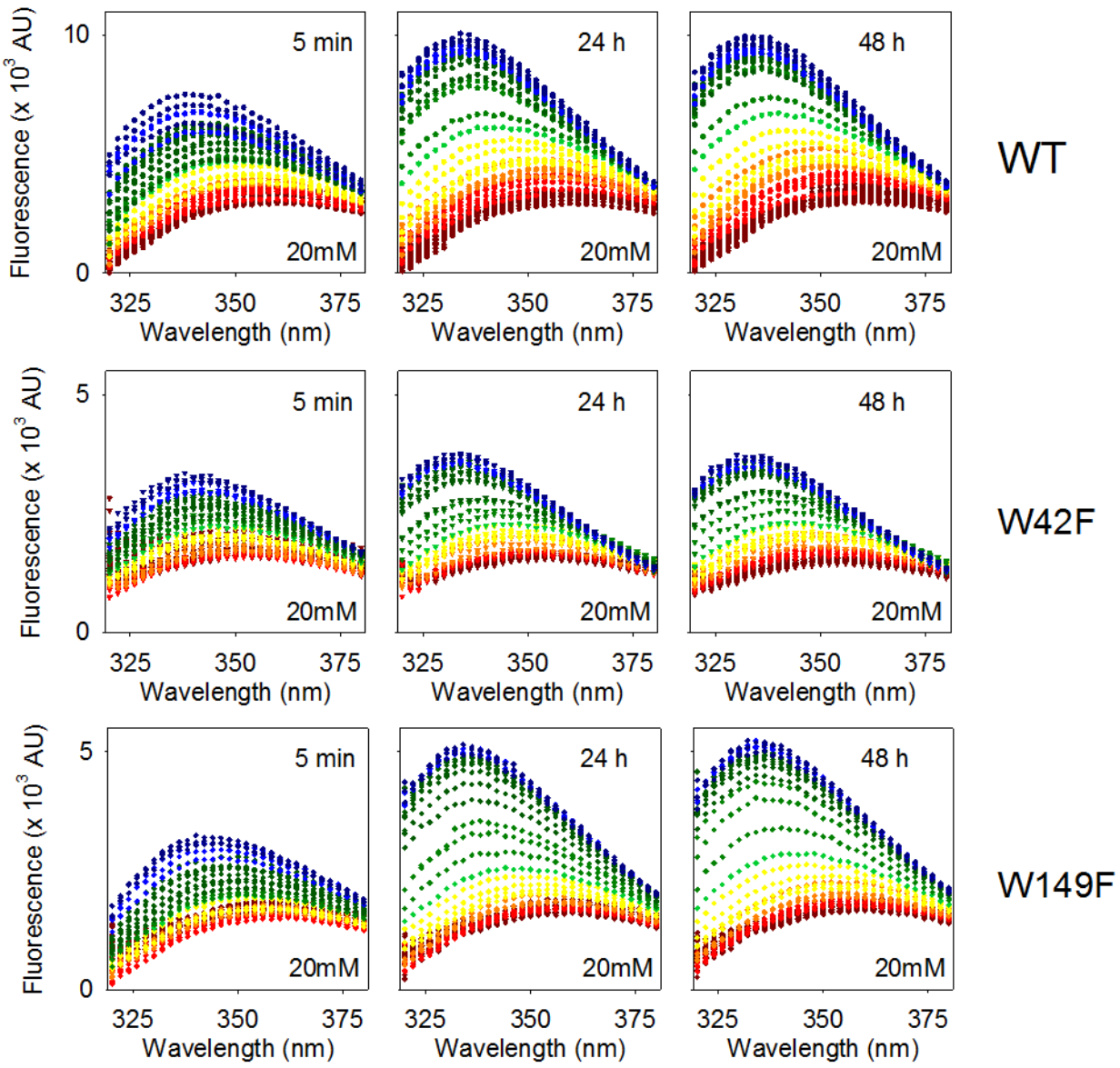
Protein	DPR	Folding experiment[a]				Unfolding experiment[a]			
		$\Delta G_{app}^{0,F}$	m_{app}	C_m [b]	Anisotropy C_m	$\Delta G_{app}^{0,U}$	m_{app}	C_m	Anisotropy C_m
		kcal/mol	kcal/mol.M	M	M	kcal/mol	kcal/mol.M	M	M
WT	700:1	-9.05 ± 1.3	2.63 ± 0.4	3.44 ± 0.0	3.97 ± 0.0	3.67 ± 0.3	-0.82 ± 0.1	4.50 ± 0.0	5.12 ± 0.1
	1750:1	-7.16 ± 1.2	2.05 ± 0.3	3.49 ± 0.0	3.84 ± 0.0	3.65 ± 0.2	-0.80 ± 0.0	4.59 ± 0.0	5.86 ± 0.0
	3500:1	-9.50 ± 0.8	2.62 ± 0.2	3.63 ± 0.0	3.69 ± 0.0	3.82 ± 0.3	-0.77 ± 0.1	4.99 ± 0.0	6.38 ± 0.5
	7000:1	-7.15 ± 0.8	2.05 ± 0.2	3.49 ± 0.0	3.53 ± 0.0	-	-	-	-
W42F	700:1	-5.65 ± 1.2	1.80 ± 0.4	3.14 ± 0.0	3.41 ± 0.0	3.71 ± 0.4	-0.85 ± 0.1	4.34 ± 0.0	4.33 ± 0.0
	1750:1	-8.04 ± 1.2	2.33 ± 0.4	3.46 ± 0.0	3.68 ± 0.0	4.35 ± 0.2	-0.93 ± 0.0	4.69 ± 0.0	4.90 ± 0.1
	3500:1	-7.45 ± 0.8	2.11 ± 0.2	3.53 ± 0.0	3.59 ± 0.0	4.22 ± 0.3	-0.83 ± 0.1	5.07 ± 0.0	6.04 ± 0.4
	7000:1	-4.21 ± 0.8	1.26 ± 0.2	3.35 ± 0.1	3.02 ± 0.0	-	-	-	-
W149F	700:1	-7.65 ± 2.0	2.28 ± 0.5	3.35 ± 0.0	3.69 ± 0.0	3.58 ± 0.2	-0.91 ± 0.1	3.95 ± 0.0	4.61 ± 0.0
	1750:1	-11.24 ± 1.2	3.18 ± 0.4	3.53 ± 0.0	3.68 ± 0.0	3.88 ± 0.3	-0.91 ± 0.1	4.25 ± 0.0	4.49 ± 0.0
	3500:1	-10.36 ± 1.2	2.91 ± 0.3	3.56 ± 0.0	3.70 ± 0.0	4.25 ± 0.4	-0.96 ± 0.1	4.44 ± 0.0	4.75 ± 0.0
	7000:1	-8.16 ± 0.6	2.13 ± 0.2	3.53 ± 0.0	3.51 ± 0.0	-	-	-	-

[a] $\Delta G_{app}^{0,F}$, $\Delta G_{app}^{0,U}$, m_{app} and C_m were derived using linear extrapolation from f_F or f_U calculations of fluorescence emission intensities at 330 nm.

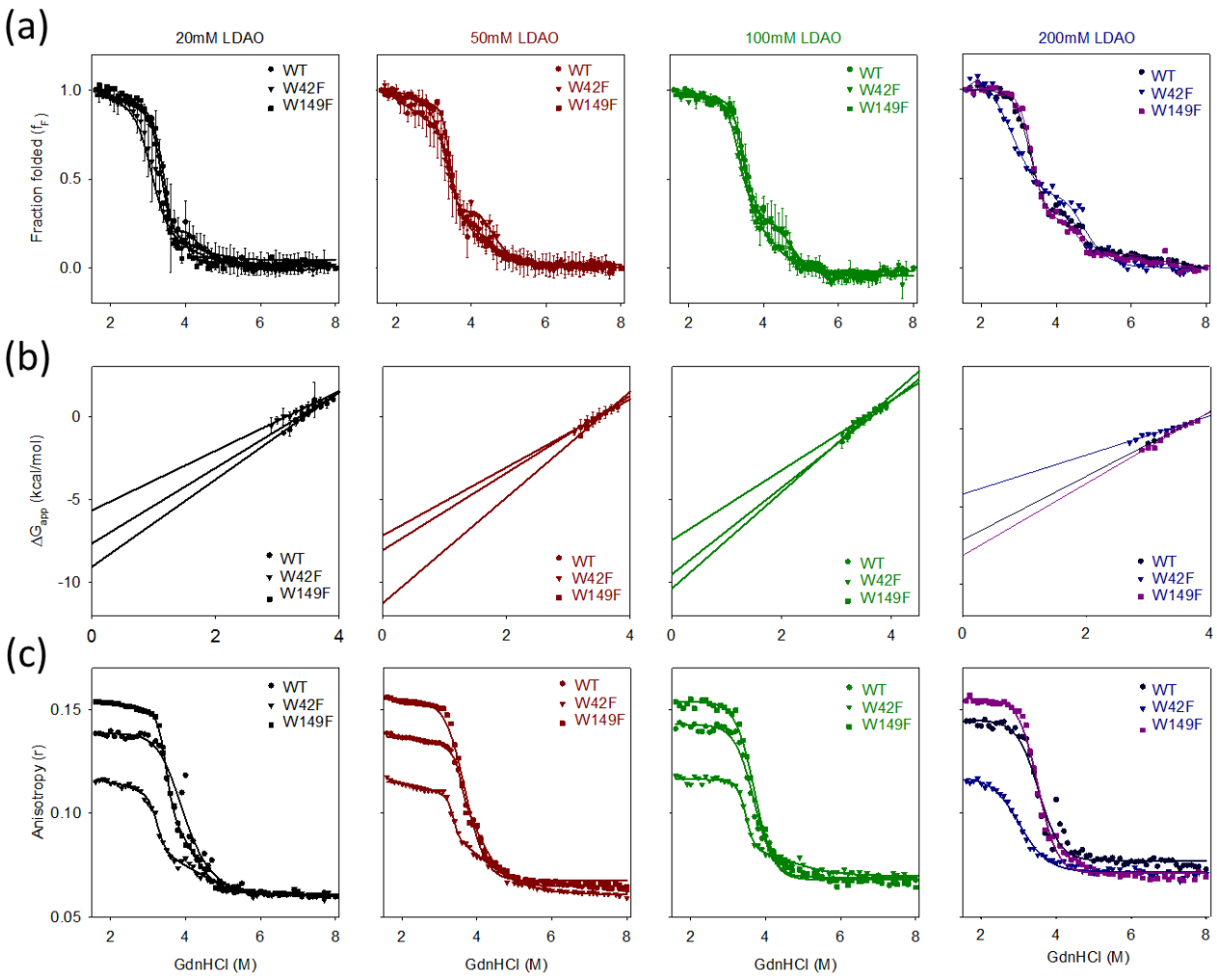
[b] In DPRs (detergent-to-protein ratios) wherein the intermediate was observed, the C_m denotes the mid-point of unfolding for conversion of the intermediate to the folded protein.



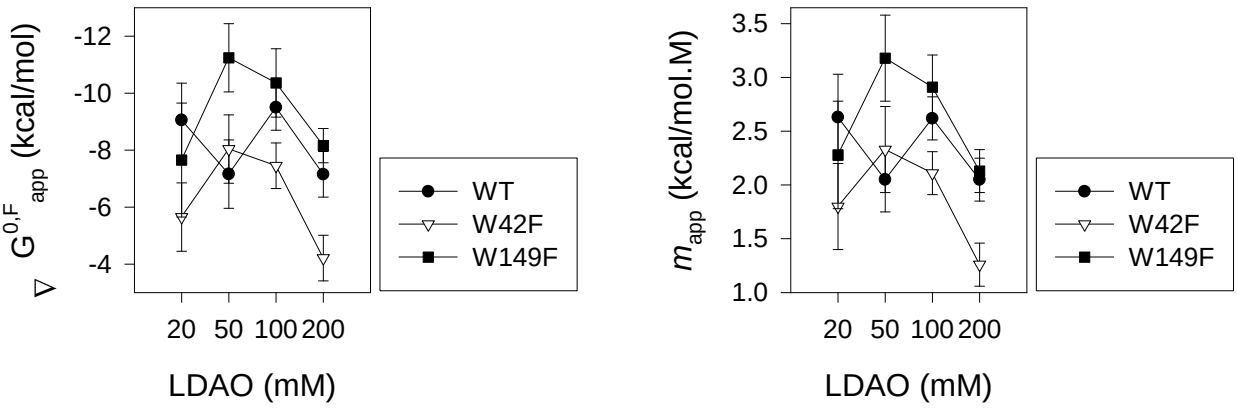
Supplementary Figure 1. (a) Acrylamide quenching measurements for Ail-W149F in 50 mM (red) and 100 mM LDAO (green), corrected for inner filter effect, still display an upward trend in the data points (linear fits are shown in black and the upward trend is highlighted using the coloured line), suggesting that static and dynamic quenching may contribute to the calculated K_{SV} for Ail-W149F in higher LDAO concentrations. To check this further, we measured the change in average lifetime (τ) with increasing GdnHCl (b). Observation of a near-linear relationship of the τ_0/τ (representative data shown in b) suggests that at high quencher concentrations, acrylamide forms stable interactions with the ground state of W42, affects the population of fluorophore molecules available for excitation at 295 nm, and gives rise to static quenching. However, we believe that the contribution of the static component to the calculated K_{SV} is not substantial, which we assess based on the <15% difference in F_0/F *versus* τ_0/τ at the highest [acrylamide], and does not, therefore, significantly influence our conclusions. We are presently unable to characterize this further, owing to experimental errors and the complex nature of protein-micelle-quencher interactions.



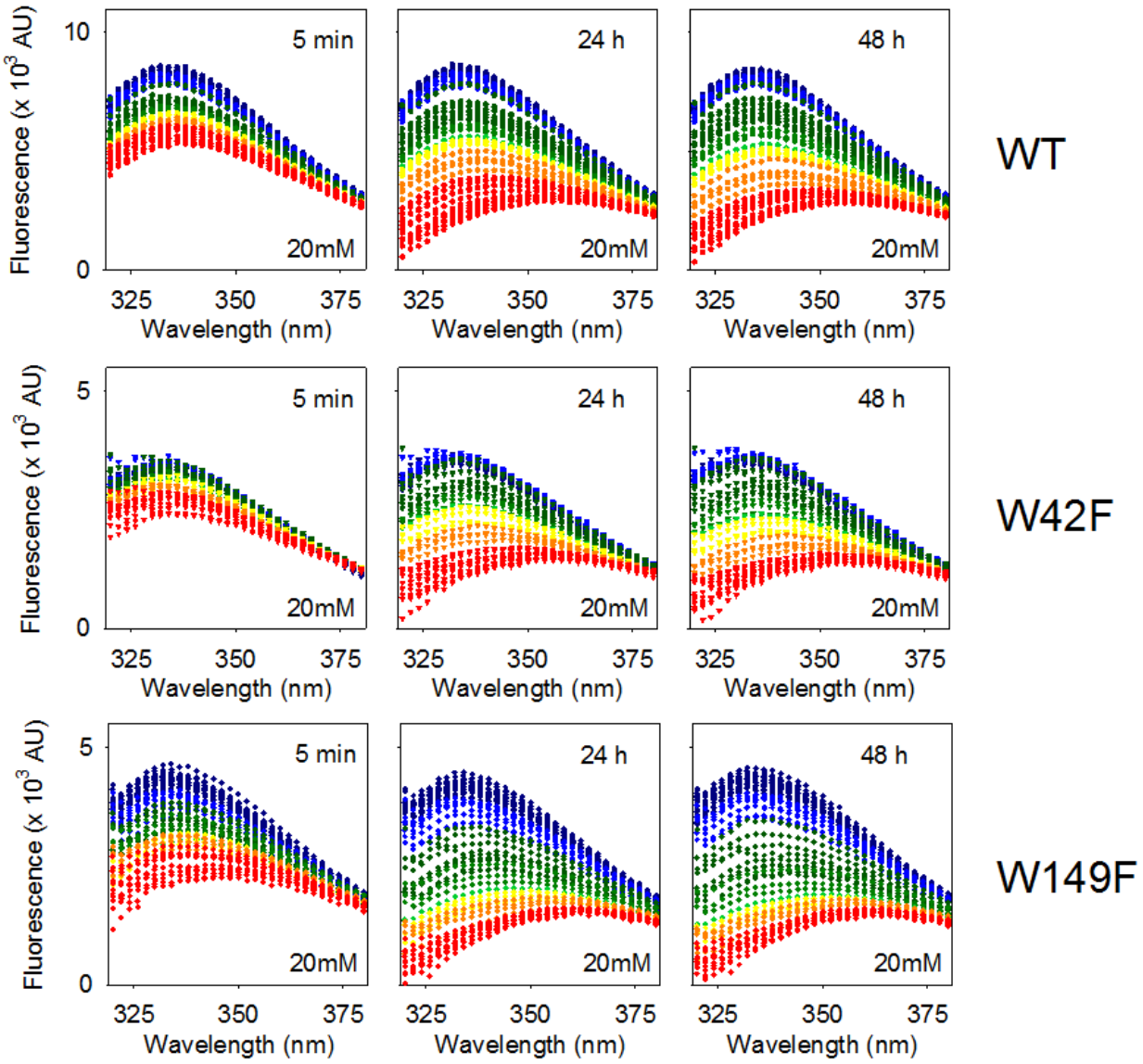
Supplementary Figure 2. Progress of the refolding reaction monitored at 5 min, 24 h and 48 h time points for Ail-WT and the two single-Trp mutants using tryptophan fluorescence. λ_{ex} = 295 nm. Shown here is the data for 20 mM LDAO as a representative example.



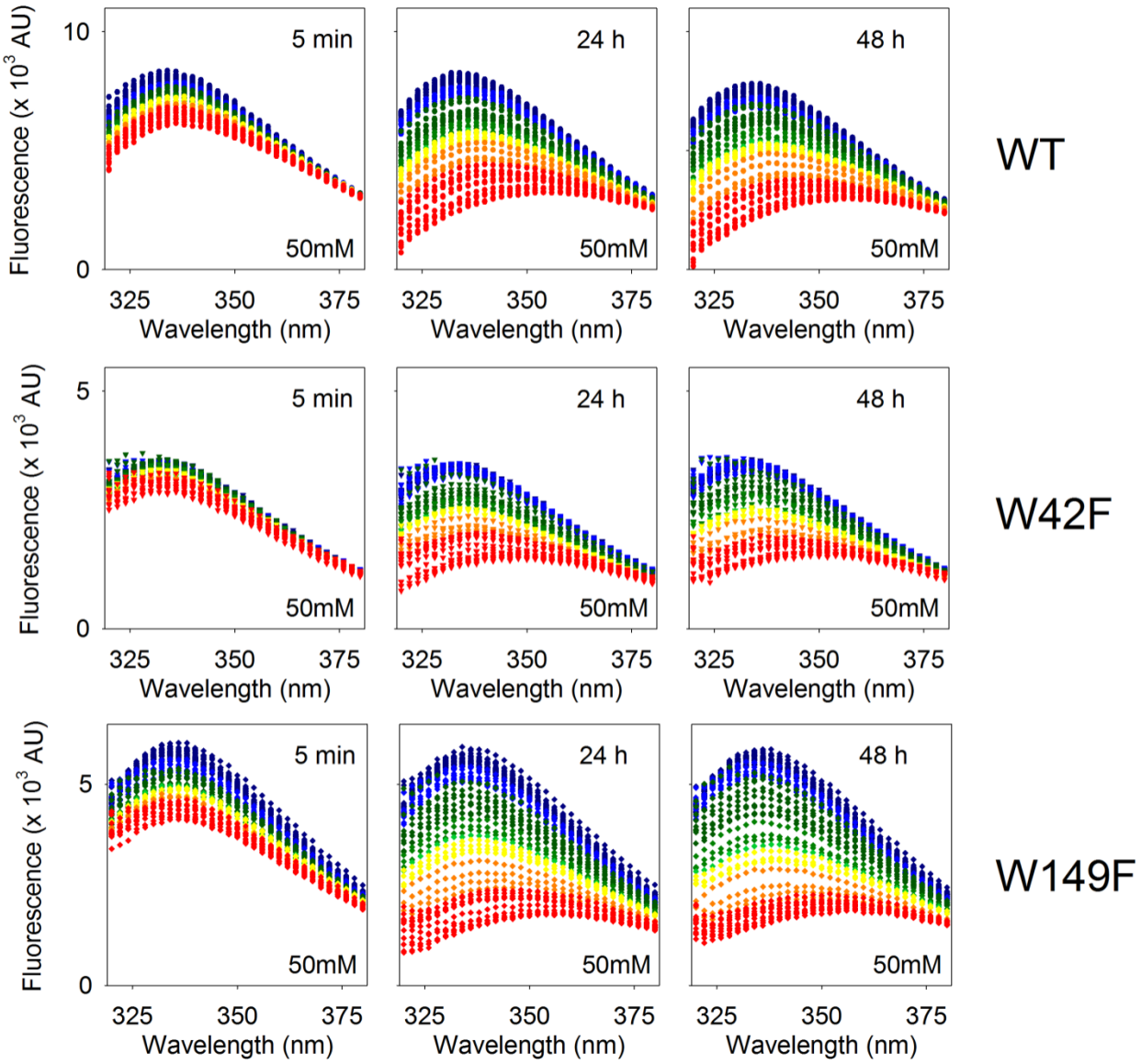
Supplementary Figure 3. (a) Fraction folded (f_F) derived from Trp emission intensity at 330 nm. (b) $\Delta G_{app}^{0,F}$ derived using linear extrapolation of the transition region. (c) Anisotropy (r) values. In all graphs, WT (○), W42F (▽) and W149F (□) are coloured according to the DPRs used (20 mM: black, 50 mM: red, 100 mM: green and 200 mM: blue/purple). Representative data are shown for anisotropy (panel c). Fits are shown as solid lines.



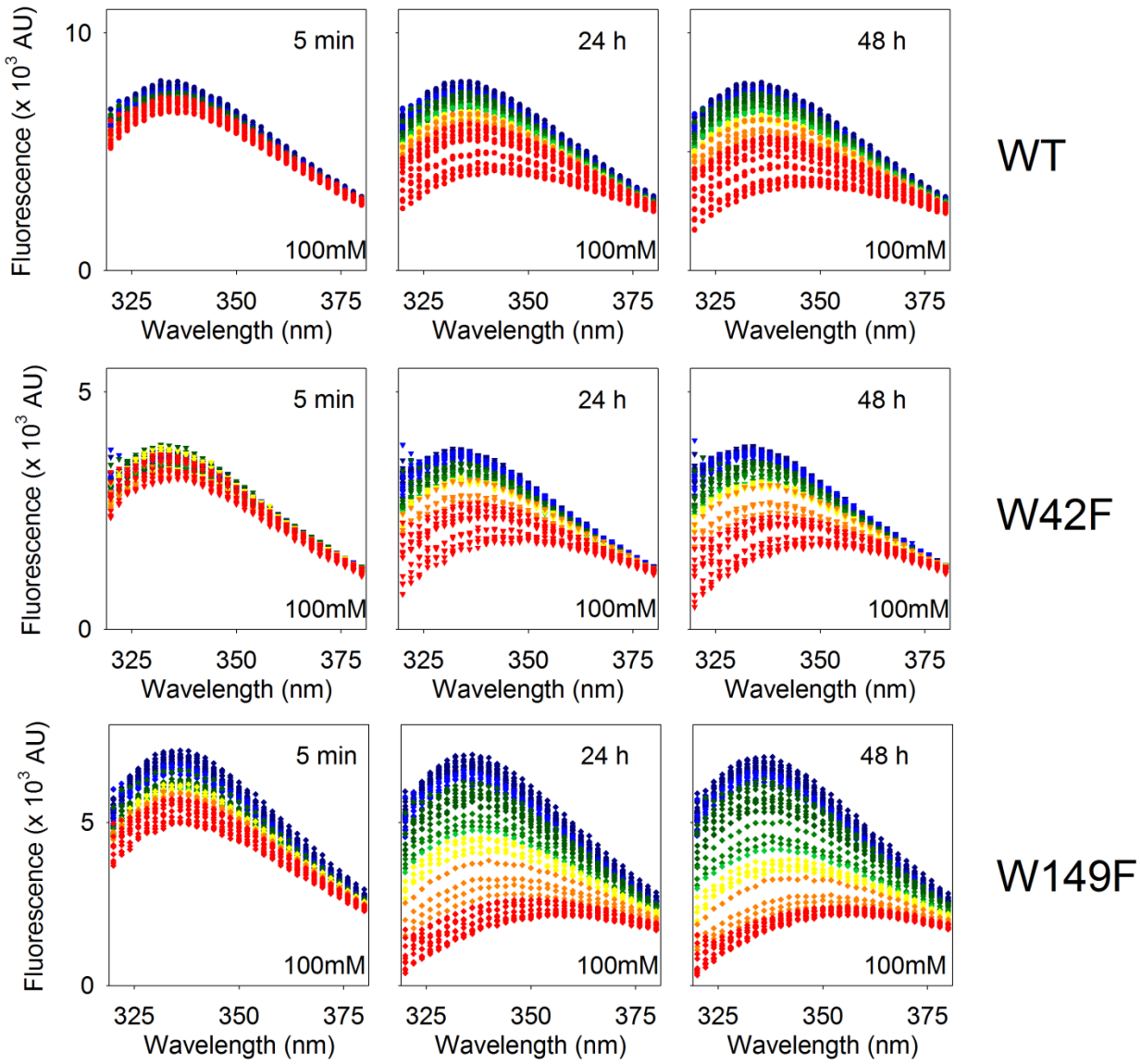
Supplementary Figure 4. Plot comparing the change in apparent folding free energy ($\Delta G_{app}^{0,F}$; left) and refolding cooperativity (m_{app} value; right) for the three proteins (WT, (●); W42F (▽); W149F (■)) in 20 - 200 mM LDAO. All three proteins show a variation in both $\Delta G_{app}^{0,F}$ and m_{app} in the order 20<50~100>200. The only deviation is seen in WT refolded in 50 mM LDAO. The highest value we could obtain for $\Delta G_{app}^{0,F}$ was -7.65 kcal/mol, which matches the $\Delta G_{app}^{0,F}$ of W42F in the same condition. We currently do not have a convincing explanation for this observation.



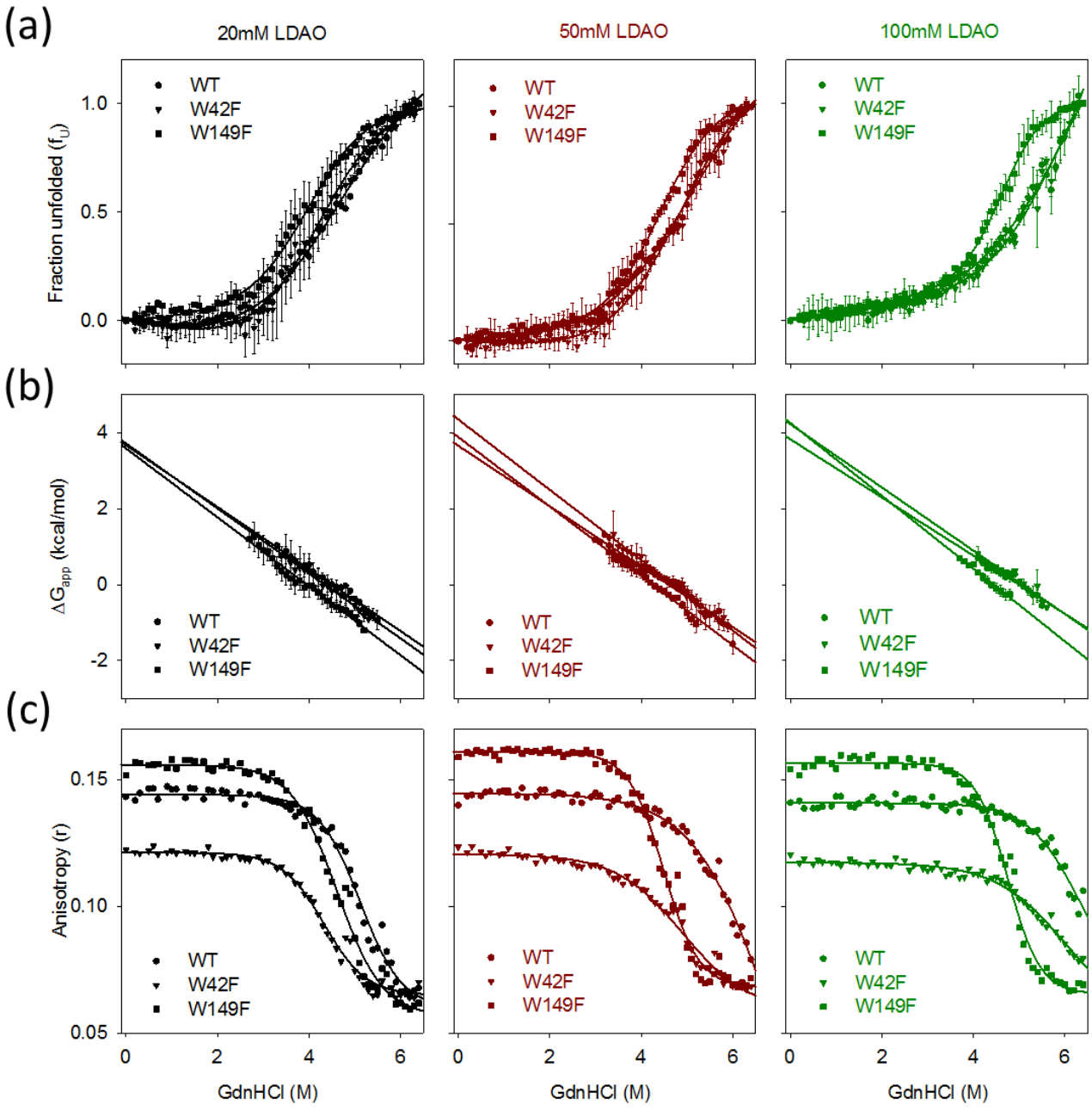
Supplementary Figure 5. Progress of the unfolding reaction monitored at 5 min, 24 h and 48 h time points for Ail-WT and the two single-Trp mutants using tryptophan fluorescence. λ_{ex} = 295 nm. Shown here is the data for 20 mM LDAO as a representative example.



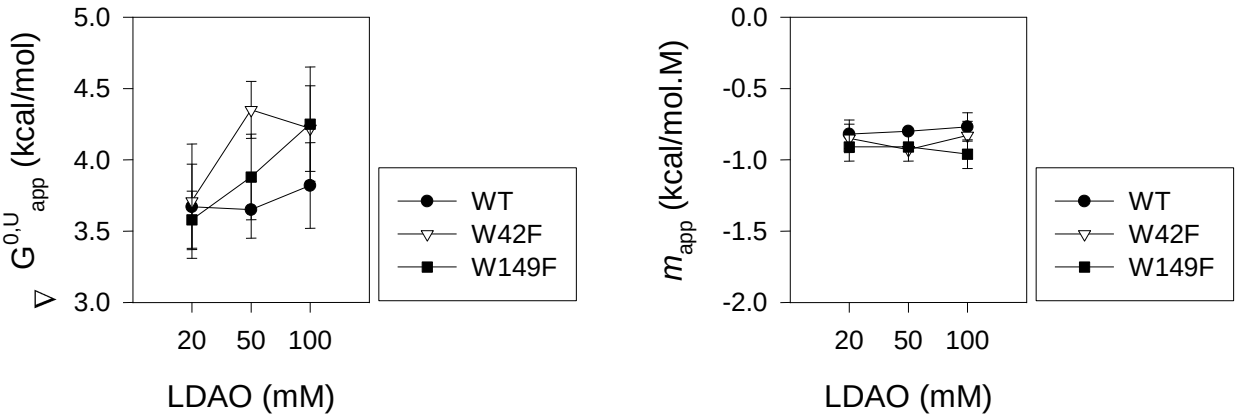
Supplementary Figure 6. Progress of the unfolding reaction monitored at 5 min, 24 h and 48 h time points for Ail-WT and the two single-Trp mutants using tryptophan fluorescence. λ_{ex} = 295 nm. Shown here is the data for 50 mM LDAO as a representative example. Note that the fluorescence intensity at 48 h for the highest GdnHCl concentrations (shown in red) are comparable to the 20 mM data (shown in Supplementary Figure 5), indicating that the protein is almost completely unfolded under these conditions.



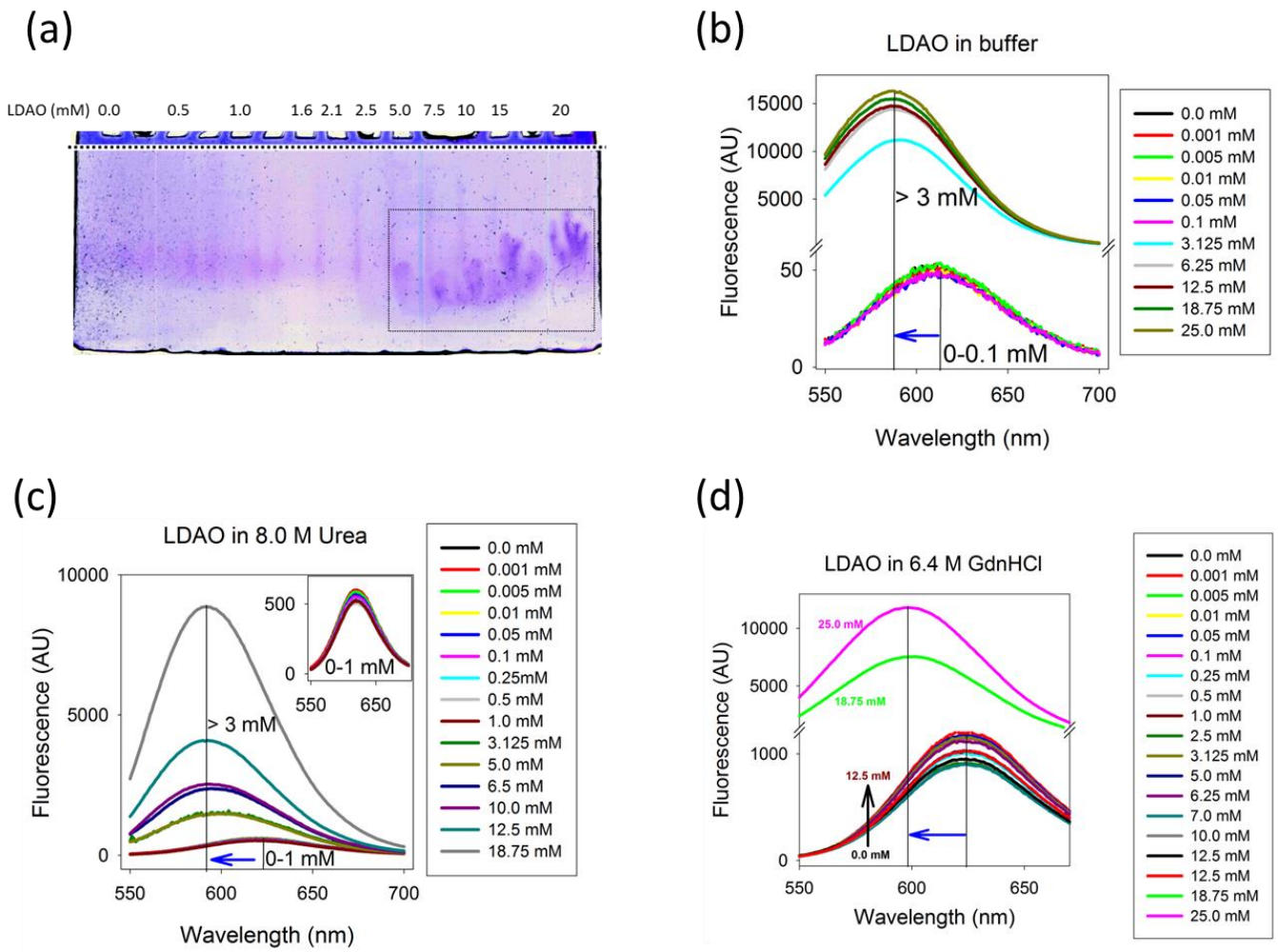
Supplementary Figure 7. Progress of the unfolding reaction monitored at 5 min, 24 h and 48 h time points for Ail-WT and the two single-Trp mutants using tryptophan fluorescence. λ_{ex} = 295 nm. Shown here is the data for 100 mM LDAO as a representative example. Note that the fluorescence intensity at 48 h for the highest GdnHCl concentrations (shown in red) are comparable to the 20 mM data (shown in Supplementary Figure 5), indicating that the protein is almost completely unfolded under these conditions.



Supplementary Figure 8. (a) Fraction unfolded (f_U) derived from Trp emission intensity at 330 nm. (b) $\Delta G_{app}^{0,U}$ derived using linear extrapolation of the transition region. (c) Anisotropy (r) values. In all graphs, WT (○), W42F (▽) and W149F (□) are coloured according to the DPRs used (20 mM: black, 50 mM: red, 100 mM: green). Error bars were obtained from at least three independent experiments. Representative data are shown for anisotropy (panel c). Fits are shown as solid lines.

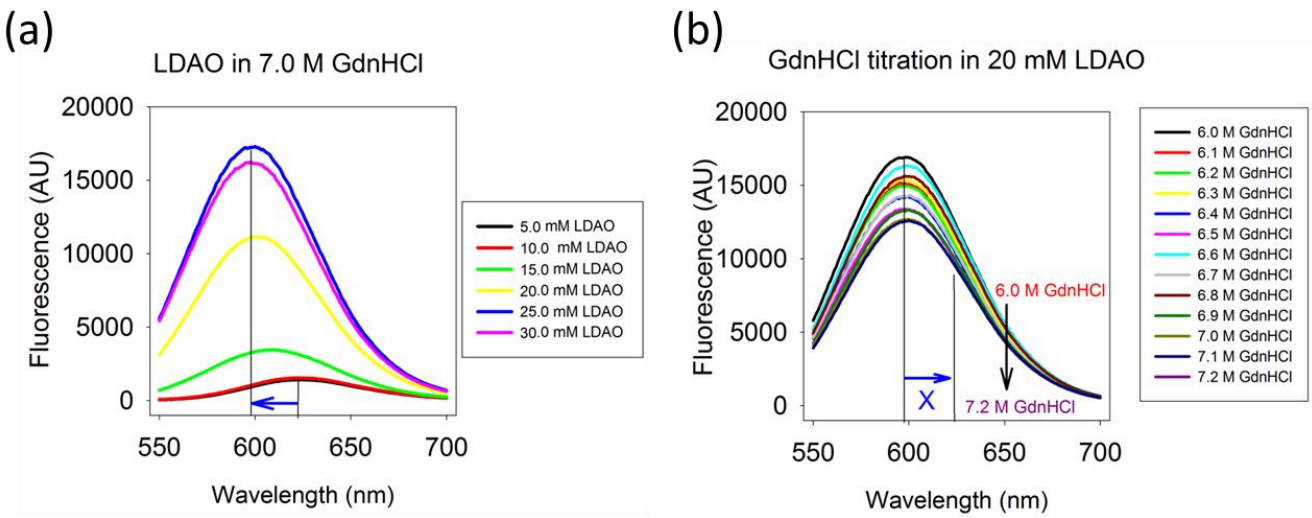


Supplementary Figure 9. Plot comparing the change in apparent unfolding free energy ($\Delta G_{app}^{0,U}$; left) and unfolding cooperativity (m_{app} value; right) for the three proteins (WT, (●); W42F (▽); W149F (■)) in 20 - 100 mM LDAO. All three proteins show a marginal variation in $\Delta G_{app}^{0,U}$ and no significant dependence on the unfolding cooperativity (m_{app} value).

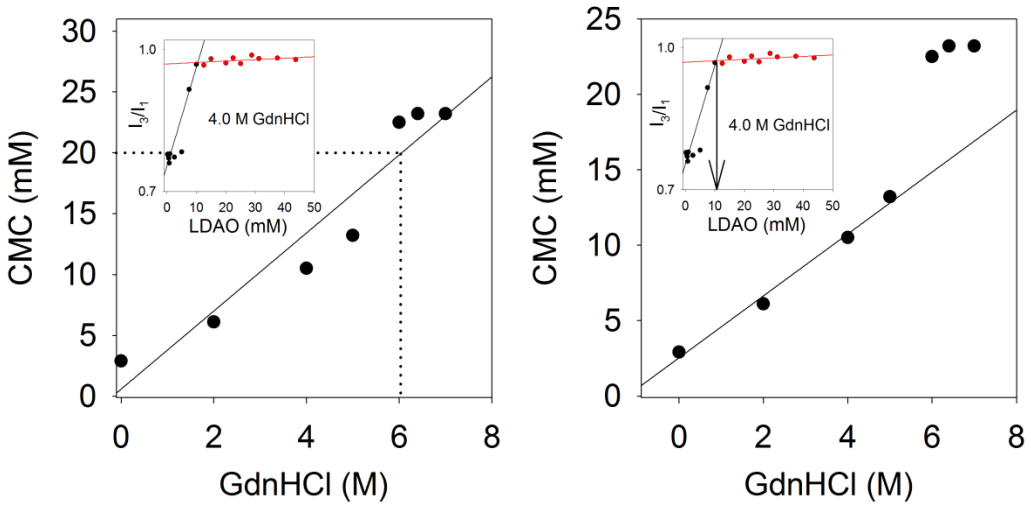


Supplementary Figure 10. Change in the CMC of LDAO upon addition of urea and GdnHCl monitored on SDS-PAGE and using SYPRO® orange fluorescence. (a) In the micellar form, LDAO can be visualized on SDS-PAGE when staining is carried out using Coomassie brilliant blue R250. The micelles appear as 'finger-like' projections that migrate close to the dye front (boxed). Solutions containing various concentrations of LDAO were prepared in 20 mM Tris-HCl pH 8.5 and were pre-treated with 7 M urea for 15-20 min at 25 °C, and examined on 15 % SDS-PAGE gels. The gel illustrates the presence of empty intact micelles in all LDAO concentrations ≥ 5 mM. The brightness/contrast of the gel has been modified to highlight the LDAO structures, since the staining efficiency of these micellar forms is poor. The dotted line separates the lower end of the gel from the wells, which have been included to demarcate the various lanes. (b-d) SYPRO® orange binds to hydrophobic regions with high affinity, and this is accompanied by an increase in fluorescence intensity of the dye. Monomeric detergent that is

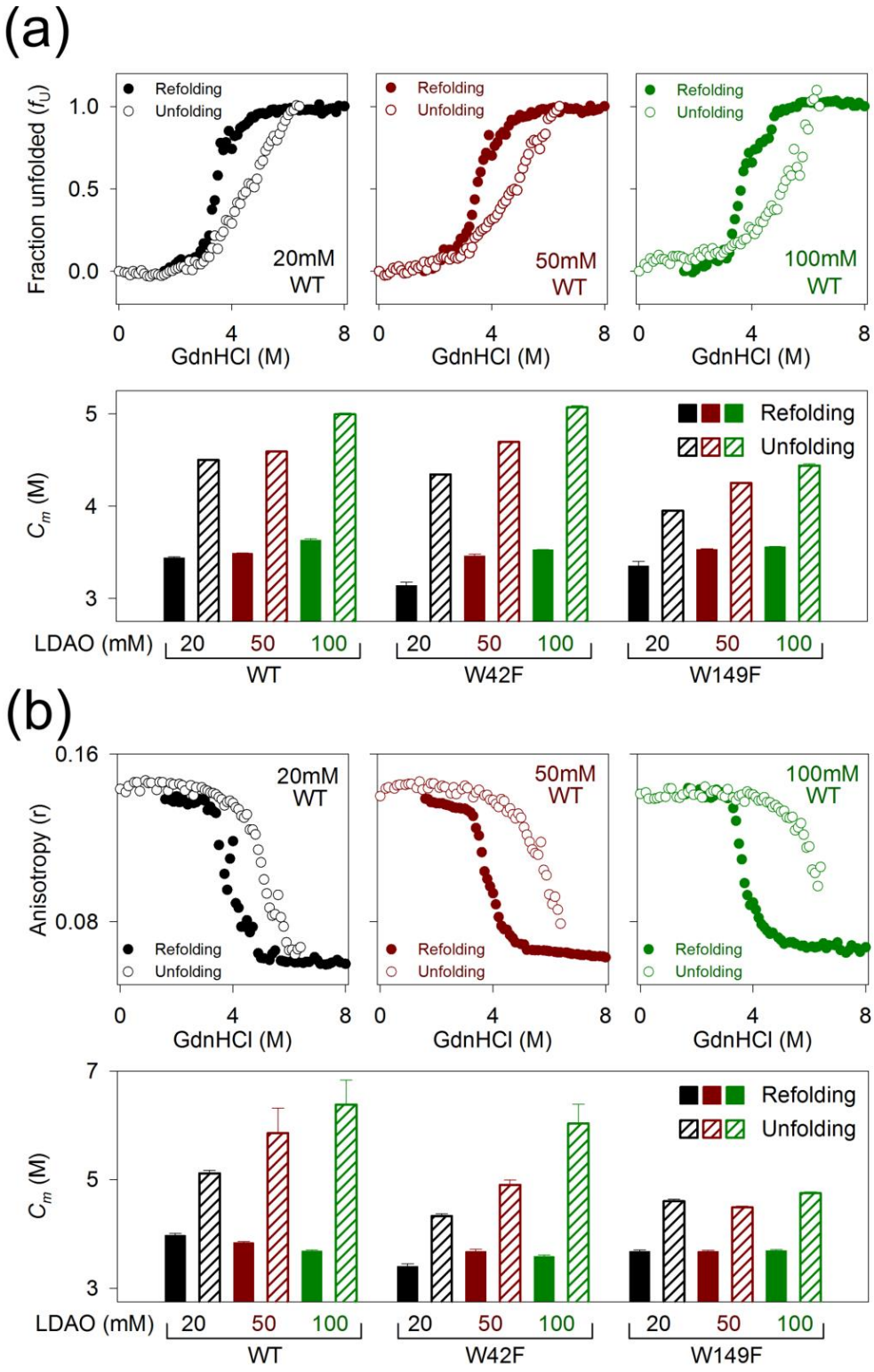
coated with denaturant molecules does not bind SYPRO® orange efficiently, and the observed fluorescence emission profile is therefore red-shifted ($\lambda_{em\text{-}max}$ = 622 nm) and of low intensity. Shown here is the observed increase in the fluorescence intensity of SYPRO® orange with change in LDAO concentration from 0.001 mM to 18.75 mM or 25 mM, in buffer (b), 8 M urea (c) and 6.4 M GdnHCl (d). A blue-shifted fluorescence ($\lambda_{em\text{-}max}$ = 587 nm in 20 mMTris-HCl pH 8.5 buffer, 590 nm in urea, and 599 nm in GdnHCl), highlighted by the drop lines and arrows, with a corresponding increase in the intensity is observed for the dye in the presence of micelles, since the latter occludes the polar solvents and provides a hydrophobic environment for the dye. All samples were incubated in the respective conditions after the addition of 20X SYPRO® orange (from a 5000X stock supplied by the manufacturer) in a 200 µl reaction, for 15-20 min at 25 °C, and wavelength scans were acquired using a $\lambda_{ex\text{-}max}$ = 480 nm and emission spectra were acquired between 550 nm and 700 nm. Based on these experiments, we have been able to derive the approximate CMCs for LDAO in urea and GdnHCl as ~5 mM and ~15 mM, respectively. The reported CMC of LDAO in water is ~1 mM. The observed CMC in buffer in our experiment is ~2 mM.



Supplementary Figure 11. (a) Change in SYPRO® orange fluorescence with increasing LDAO concentration in 7.0 M GdnHCl (shown here is data from a representative experiment; see the legend of Supplementary Figure 10 for details of buffer conditions and experimental set-up). Increase in fluorescence intensity accompanied by a blue-shifted emission is observed beyond 15 mM LDAO, suggesting that LDAO micelle formation in the presence of 7.0M GdnHCl occurs only beyond ~15 mM LDAO. (b) Change in the SYPRO® orange fluorescence monitored with increasing GdnHCl concentration from 6.0 M to 7.2 M, in 20 mM LDAO. The prominent red shift in SYPRO® fluorescence accompanied by reduction in emission intensity (indicated by drop lines) is absent, suggesting that some micellar structures persist at the highest GdnHCl concentrations used in our experiments. Furthermore, based on the SYPRO® orange fluorescence intensity in 25 mM LDAO (shown in (a)), it is evident that dissolution of micelles formed in 50 mM and 100 mM LDAO will require very high GdnHCl concentrations (>> 7.0 M), which cannot be practically achieved.

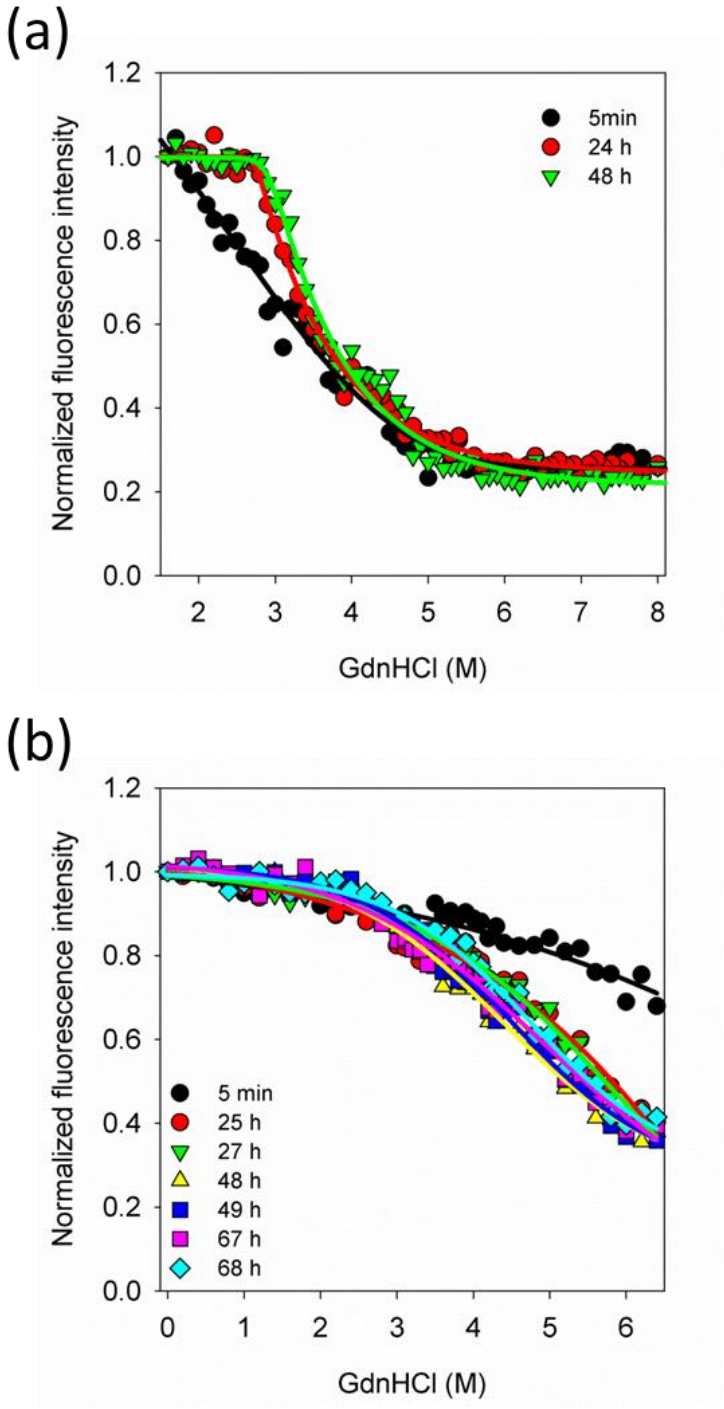


Supplementary Figure 12. Monitoring the change in CMC of LDAO with GdnHCl, using pyrene fluorescence, as reported earlier.[1] A representative dataset showing how the CMC was determined at 4.0 M GdnHCl is provided as inset. The left graph illustrates the fit to all data points and the right graph illustrates the change in CMC extrapolated after excluding the data between 6-7 M. We consistently obtained a CMC of ~22 mM between 6-7 M GdnHCl, which indicated that the non-linearity of the CMC between this region was not an experimental artefact. This is not in agreement with our SYPRO® orange measurements (Supplementary Figures 10 and 11). We currently do not have an explanation for this deviation. Nevertheless, for the purpose of our experiments, it was important to address if the LDAO micelles were dissolved prior to the protein unfolding event. Dotted lines in the left graph highlight the lowest LDAO concentration used in our experiments. In 20 mM LDAO, micelles are disrupted at GdnHCl concentrations ≥ 6.0 M. Protein unfolding in 20 mM LDAO is completed by 6.0 M GdnHCl, and display C_m values in the range of 3.9 – 4.5 M. In higher LDAO concentrations (50 mM and 100 mM), considerably high GdnHCl (>> 7.0 M) would be required, which cannot be practically achieved. Nevertheless, we still observe Ail unfolding in these LDAO concentrations (see Supplementary Figures 5-7). Hence, we believe that Ail unfolding can be nucleated even in the presence of intact micelles.



Supplementary Figure 13. Hysteresis in Ail arises from variations in barrel-micelle interaction efficacy. (a, top) Comparison of the GdnHCl-mediated refolding (filled circles) and unfolding (open circles) curves, shown here for Ail-WT. Data were obtained from unfolded fractions (f_U)

derived from Trp fluorescence, in 20 mM (black), 50 mM (red) and 100 mM (green) LDAO. The mean values from two independent experiments are provided here, without error bars. C_m values obtained for the three proteins from these f_U plots, for both refolding (solid fills) and unfolding (diagonal stripes) data, are shown as a function of increasing LDAO concentration (a, bottom). A clear variation is seen with increasing LDAO, in all the three proteins, for the unfolding C_m, whereas the refolding C_m values are less sensitive to changes in DPR. (b, top) Representative Trp anisotropy values obtained for the GdnHCl-mediated refolding (filled circles) and unfolding (open circles) of WT Ail. (b, bottom) C_m values show DPR dependence only for the unfolding curves, while the refolding data is DPR independent, as observed from f_U calculations. Note that while the f_U data for the unfolding experiment tends to 1.0 in 100 mM LDAO (a, bottom), the corresponding anisotropy values do not reach ~0.06 (for a fully unfolded protein), suggesting that unfolded Ail probably binds substantially to LDAO. A similar colour scheme is retained in all datasets. Error bars denote goodness of fits.



Supplementary Figure 14. Progress of the refolding and unfolding reactions of Ail, shown here for the W42F protein in 50 mM LDAO as a representative example. (a) Folding reaction. (b) Unfolding reaction. Values are normalized to the 0 M data.

References:

1 Andersen, K. K., Wang, H. & Otzen, D. E. A kinetic analysis of the folding and unfolding of OmpA in urea and guanidinium chloride: single and parallel pathways. *Biochemistry* **51**, 8371-8383 (2012).