CHAPTER 4

Tryptophan fluorescence quenching by brominated lipids

4.1 INTRODUCTION

The structure and dynamics of biological macromolecules have been widely studied with fluorescence quenching. The accessibility of tryptophan in proteins, and thus its solvent exposure, has been probed by polar and nonpolar quenchers (London & Feigenson, 1981; Lakowicz, 1999). The use of quenchers covalently linked to phospholipids at specific depths such as nitroxide or bromine-labeled lipids, have been used to approximate the positions of protein fluorophores, such as tryptophan, in the bilayer (London & Feigenson, 1981; Markello et al., 1985). X-ray diffraction has revealed the positions of the bromines in the bilayer, making brominated lipids particularly useful (Markello et al., 1985; McIntosh & Holloway, 1987).

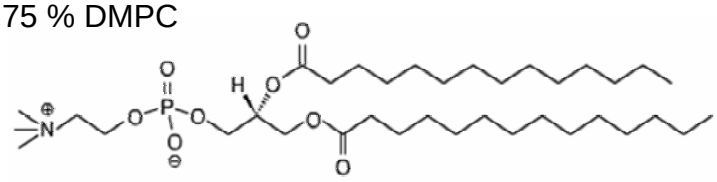
Mixed vesicles of DMPC and DOPC with brominated lipids have been used to study the location of the Trps in folded OmpA (Rodionova et al., 1995) and during the course of the OmpA folding reaction using single tryptophan mutants (Kleinschmidt & Tamm, 1999). Characterization of the adsorbed/partially inserted and folded forms of wt-OmpA into DMPC vesicles was performed using 20% brominated lipids (Rodionova et al., 1995). Gel shift assays showed that this concentration did not affect folding of OmpA into Br-lipid/DMPC mixed vesicles as evidenced by a 30 kDa band. However the experiments were done using wt-OmpA and was thus an average of the Trp fluorescence.

Tamm and others also used brominated lipids to report the collective movement of the five Trps as OmpA folded into brominated lipids using time-resolved distance determination by tryptophan fluorescence quenching (TDFQ) (Kleinschmidt & Tamm, 1999). This technique involved measuring the steady-state fluorescence over the course of folding and was used to monitor Trp movement at temperatures from 2 ^{o}C to 40 ^{o}C.

Quenching for the tryptophans was observed from the start of the refolding experiments, therefore verifying that OmpA adsorbs to the bilayer surface within the mixing dead-time of 1-2 min. This technique revealed three membrane bound intermediates formed during folding. TDFQ was further utilized to study single-tryptophan mutants during the course of folding. Results revealed that OmpA inserts and folds in lipid bilayers by a concerted mechanism and that the 4 β-hairpins traverse the bilayer synchronously (Kleinschmidt et al., 1999).

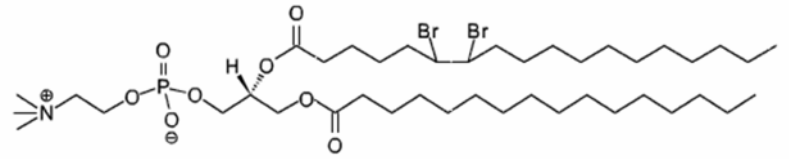
In this Chapter, we describe both steady-state and time-resolved fluorescence to study the fluorescence quenching of Trp mutants folded in mixed vesicles of DMPC and di-brominated (DiBr) lipids in a 3:1 molar ratio. The purpose of these experiments was to further characterize the Trp locations within the bilayer in the full-length and truncated proteins. We used two different DiBr attached to different positions along the sn-2 acyl chain of the phospholipids (Figure 4.1).

75 % DMPC



+

25 % 6,7-DiBr



or

25 % 11,12-DiBr

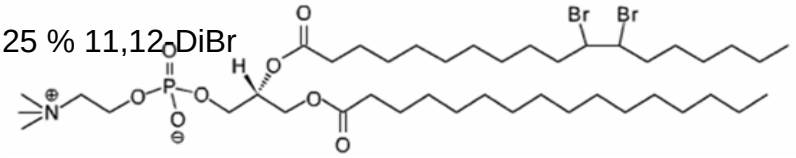


Figure 4.1. Structures of DMPC, 1-palmitoyl-2-stearoyl (6-7) dibromo-*sn*-glycero-3-phosphocholine (6,7-DiBr), and 1-palmitoyl-2-stearoyl (11-12) dibromo-*sn*-glycero-3-phosphocholine (11,12-DiBr). Mixed vesicles of DMPC/6,7-DiBr and DMPC/11,12-DiBr were prepared with a 0.25 molar fraction of DMPC:DiBr lipids.

4.2 EXPERIMENTALS

Preparation of brominated vesicles are found in section 2.2. Steady-state and time-resolved fluorescence measurements were performed at 30 ^{o}C as described in section 3.2. Quenched and unquenched data for both lipids were obtained on the same day to ensure that the relative differences could be compared. Protein concentrations ranged from ~2.1 – 3.8 μM and lipid concentrations were ~1.5 mM.

4.3 RESULTS AND DISCUSSION

Fluorescence quenching by heavy atoms such as iodine and bromine possibly promotes spin-orbit coupling of the singlet excited state and the heavy atom, leading to intersystem crossing to the excited triplet state (Kasha, 1952). The result is a decrease in the average excited-state lifetime and emission quantum yield. Brominated lipids were utilized to study the extent of Trp fluorescence quenching for each Trp residue in the full-length and truncated mutants. Steady-state (Figure 4.2, 4.3) and time-resolved (Figure 4.4, 4.5) fluorescence spectra displayed fluorescence quenching by the bromines. Bromines in the 6,7-DiBr lipids are located close to the polar head group while 11,12-DiBr lipids are located deeper in the bilayer. X-ray diffraction revealed the average positions of the bromines from the center of the lipid bilayer to be 11.0 Å and 6.5 Å for 6,7-DiBr and 11,12-DiBr, respectively (McIntosh & Holloway, 1987).

Steady-state quenching was determined by obtaining the quenched to unquenched ratio of the emission maxima (Table 4.1). From time-resolved data, quenching rates were obtained by dividing excited-state decays of protein in DiBr vesicles with those of protein in DMPC vesicles. Overlay of the quenching rates for each of the DiBr vesicles revealed

that the W102 and W102t mutants are the least quenched Trp for the full-length and truncated proteins (Figure 4.6, 4.7). The unquenched amplitude was ~85-95%. This suggests that W102 is the furthest away from the quenchers, which is reasonable since this is the only residue that faces the protein pore and does not interact with the lipid bilayer. The W57 and W57t position is the next least quenched with ~70-80% unquenched population. In contrast, W7 and W7t are the most quenched (~50%) Trp in both lipids, indicating that W7 is closest to both the 6,7 and 11,12 positions and therefore W7 is the closest to the hydrocarbon core. If it were the furthest from the hydrocarbon core, we would observe smaller quenching by 11,12-DiBr, which is not the case here. Slightly slower, although essentially similar, quenching rates to W7 and W7t, are observed for W15t and W143t in 6,7-DiBr and for both full-length and truncated W15 and W143 mutants in 11,12-DiBr.

Superposition of quenching rates for mutants in 6,7-DiBr and 11,12-DiBr showed that in general 6,7-DiBr is the more effective quencher (Figure 4.8). This indicates that the Trp residues are located closer to the polar head groups rather than the bilayer center. Previous research from steady-state fluorescence with brominated lipids also observed that W7 was the most quenched Trp residue while W102 was the least quenched. In addition, the most effective quencher was 9,10-DiBr, followed by 6,7-DiBr and 4,5-DrBr. The least effective quencher was 11,12-DiBr, which is the closest to the bilayer center (Kleinschmidt et al., 1999). The results described here and reported observations are consistent with the structures of OmpA where the Trps are located near the water-bilayer interface (Pautsch & Schulz, 1998; Arora et al., 2001). Although some of the truncated mutants appear to be quenched differently from full-length mutants, differences are too

small to conclude that the Trp locations of the truncated protein are different from Trp locations in the full-length protein (Figure 4.9). The slight differences between full-length and truncated data are consistent with conclusions described in Chapter 3; the C-terminal domain minimally perturbs the transmembrane environment of the Trp residues.

Multiple data sets of steady-state fluorescence were not very consistent and did not produce clear results regarding which Trp was the most or least quenched. It is possible that the brominated lipids may not have been uniformly dispersed in the vesicles. In addition, it is possible that lateral diffusion of the lipids within the vesicles are occurring since the vesicles are fluid at high temperatures. Data from fluorescence energy transfer kinetics, described in Chapter 6, also reveal that about half the protein does not fold to the native structure and therefore would lead to incomplete quenching of Trp fluorescence and produce discrepancies observed in different data sets.

Despite ambiguous fluorescence data and unknown factors to consider when interpreting the data, time-resolved fluorescence data were slightly more informative but only provided information that has already been reported (Kleinschmidt et al., 1999). Since results did not appear too insightful, measurements at 15 ^{o}C were not made although they may reveal some information about the adsorbed species. Studies by Radionova and coworkers (1995) attempted to characterize the adsorbed species and showed that at 10 ^{o}C, quenching of the wt-OmpA fluorescence by 9,7-DiBr-DMPC was the greatest compared to quenching by 4,5 and 11,12-DiBr-DMPC. Since the bromines in 9,7-DiBr are located in the middle of the lipid chain, it was concluded that the Trps are actually partially buried in the bilayer at this adsorbed state. However, it is possible that the presence of the DiBr in the DMPC vesicles may have changed the lipid properties and

the gel-liquid transition temperature. The bromines may have also introduced defects to the surface of the vesicles in the gel state since they are slightly longer than DMPC and thus allow OmpA to become partially inserted. Furthermore, it was not clear whether the starting vesicle solution was pre-equilibrated at low temperature prior to protein injection or whether the protein was folded into warm vesicles and then incubated at low temperatures.

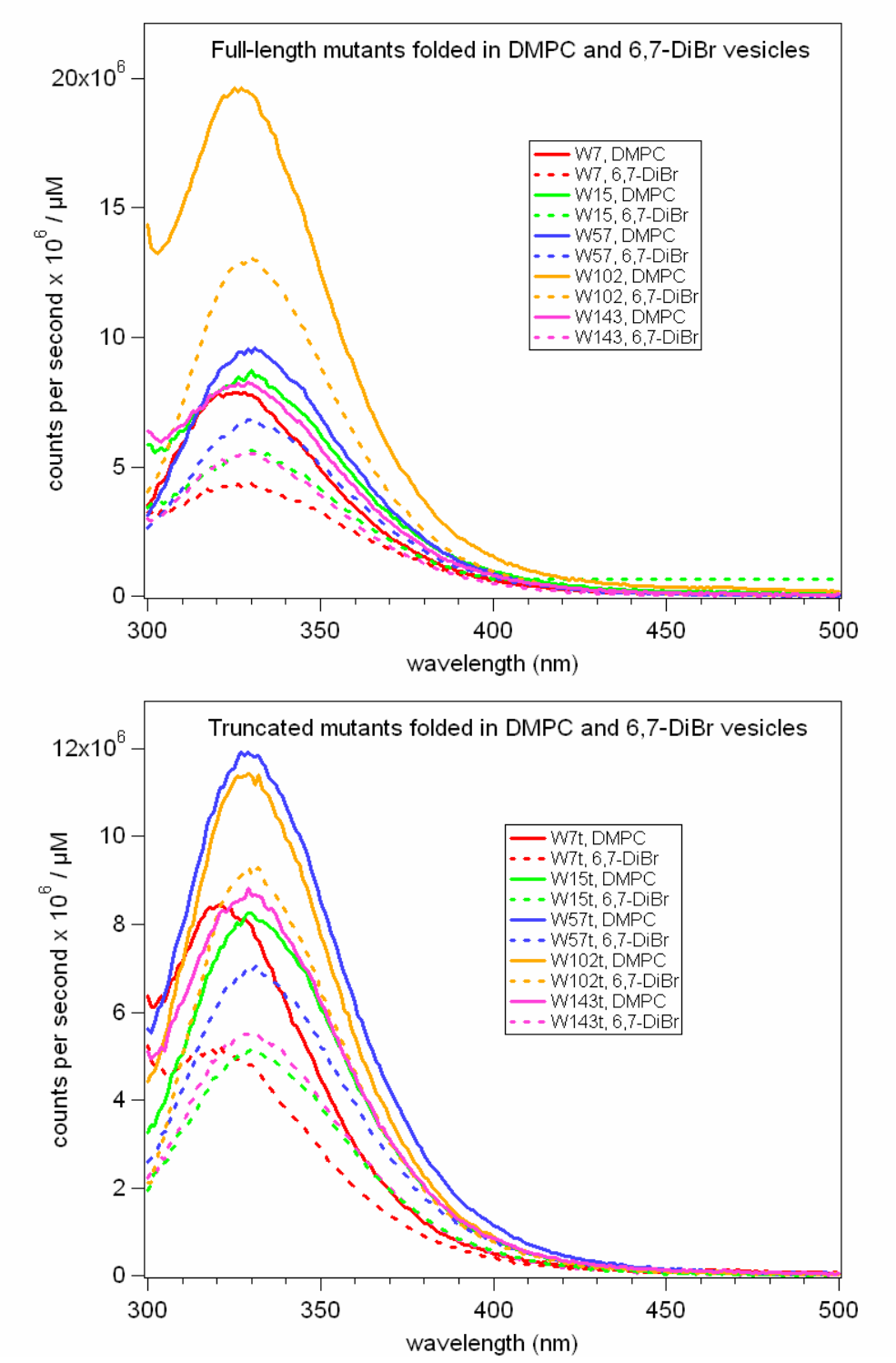


Figure 4.2. Steady-state fluorescence of full-length (top) and truncated (bottom) mutants in DMPC (solid lines) and 6,7-DiBr (dashed lines) vesicles

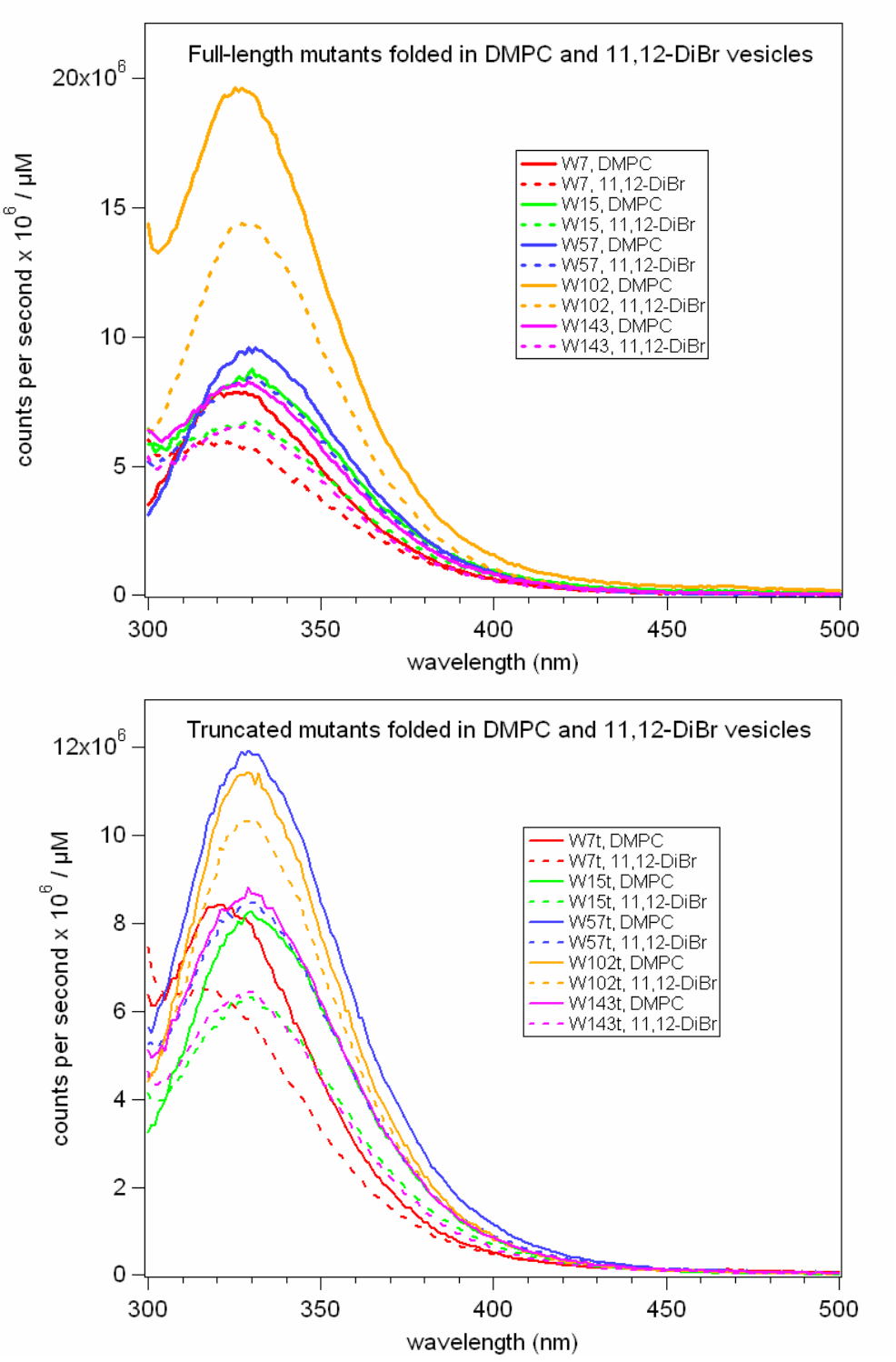


Figure 4.3. Steady-state fluorescence of full-length (top) and truncated (bottom) mutants in DMPC (solid lines) and 11,12-DiBr (dashed lines) vesicles

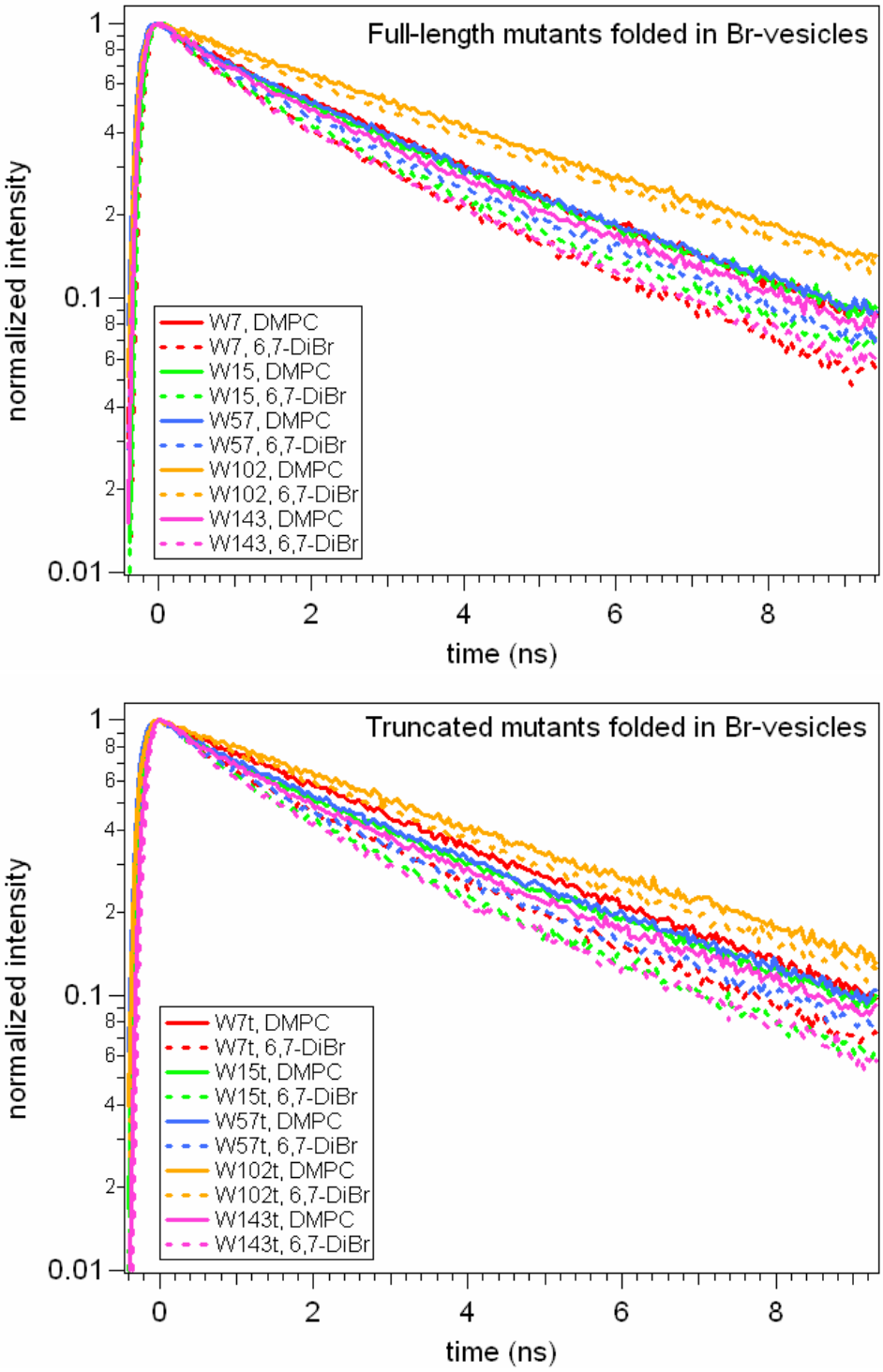


Figure 4.4. Fluorescence decays of full-length (top) and truncated (bottom) Trp mutants folded in DMPC vesicles (solid lines) and 6,7-DiBr vesicles (dashed lines) at 30 ^{o}C.

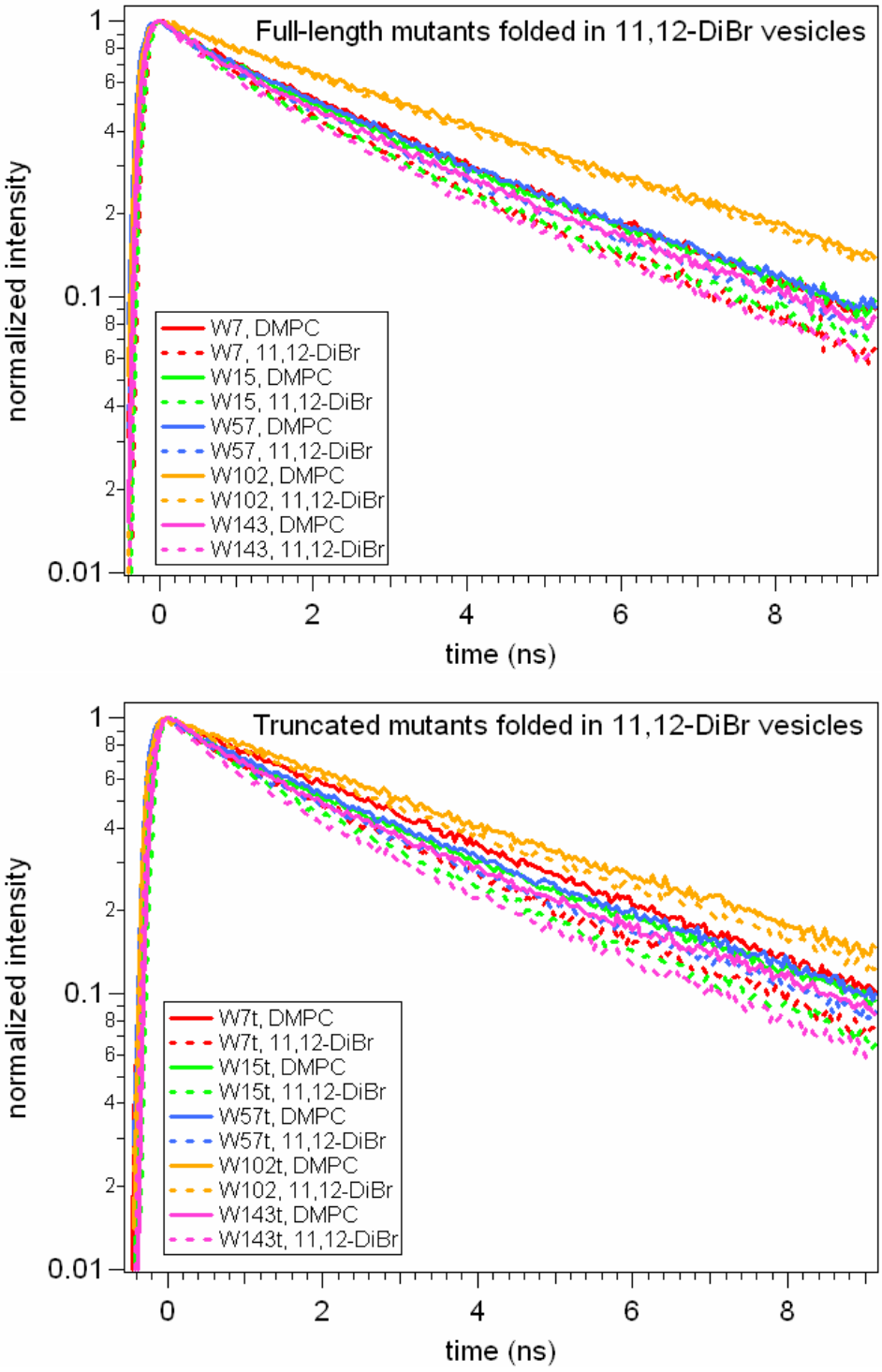


Figure 4.5. Fluorescence decays of full-length (top) and truncated (bottom) Trp mutants folded in DMPC vesicles (solid lines) and 11,12-DiBr vesicles (dashed lines) at 30 ^{o}C.

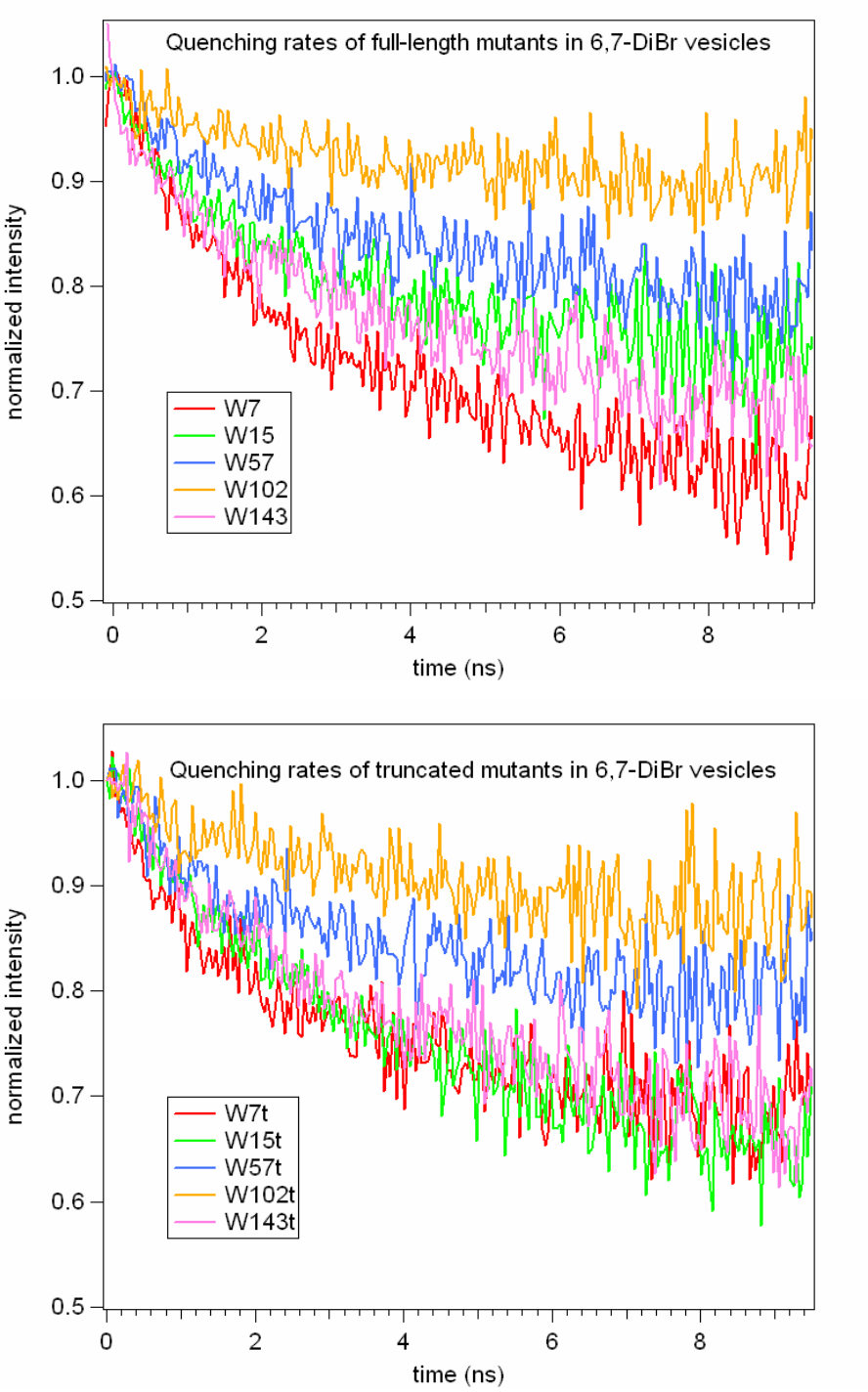


Figure 4.6. Fluorescence quenching rates of full-length (top) and truncated (bottom) mutants in 6,7-DiBr vesicles at 30 ^{o}C.

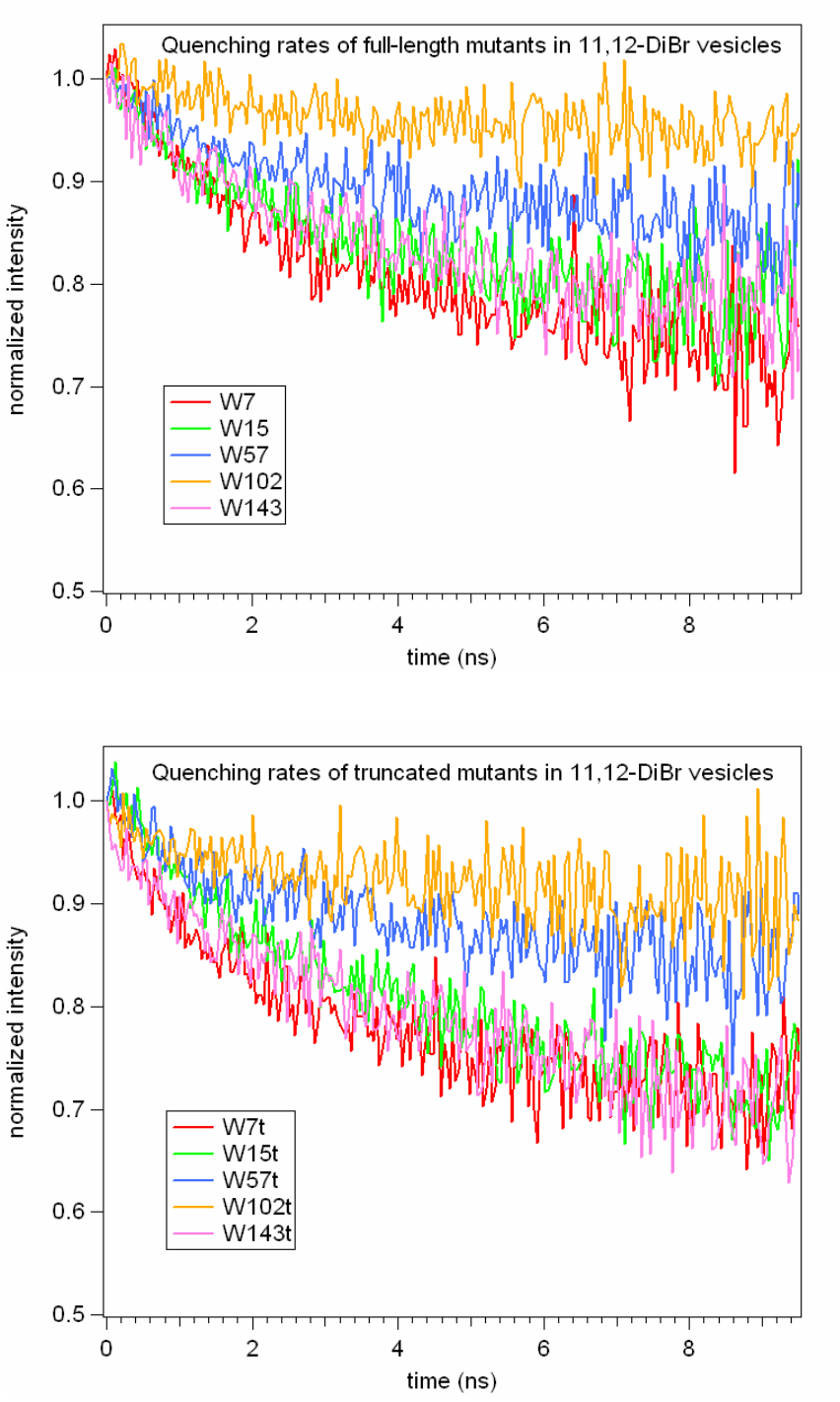


Figure 4.7. Fluorescence quenching rates of full-length (top) and truncated (bottom) mutants in 11,12-DiBr vesicles at 30 ^{o}C.

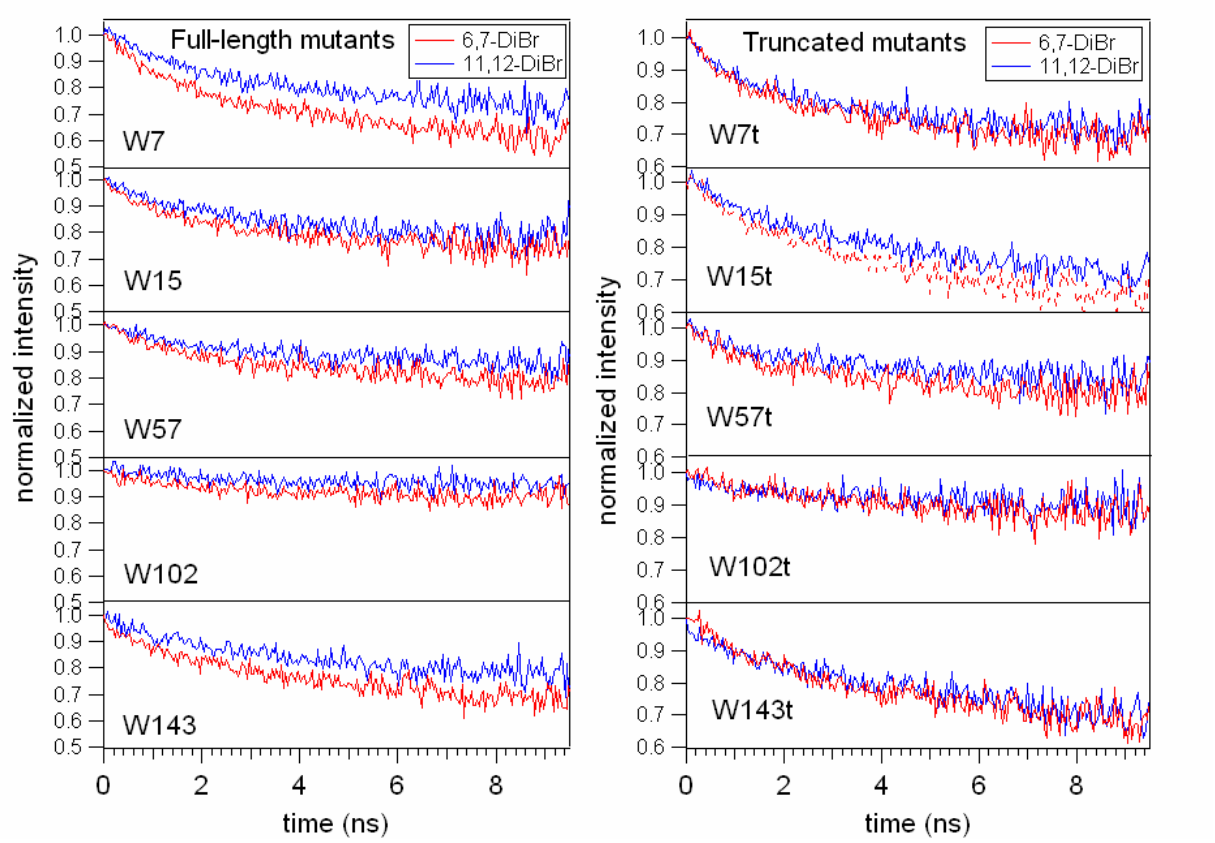


Figure 4.8. Overlay of the quenching rate of full-length mutants (left) and truncated mutants (right) folded in the two different DiBr lipid positions.

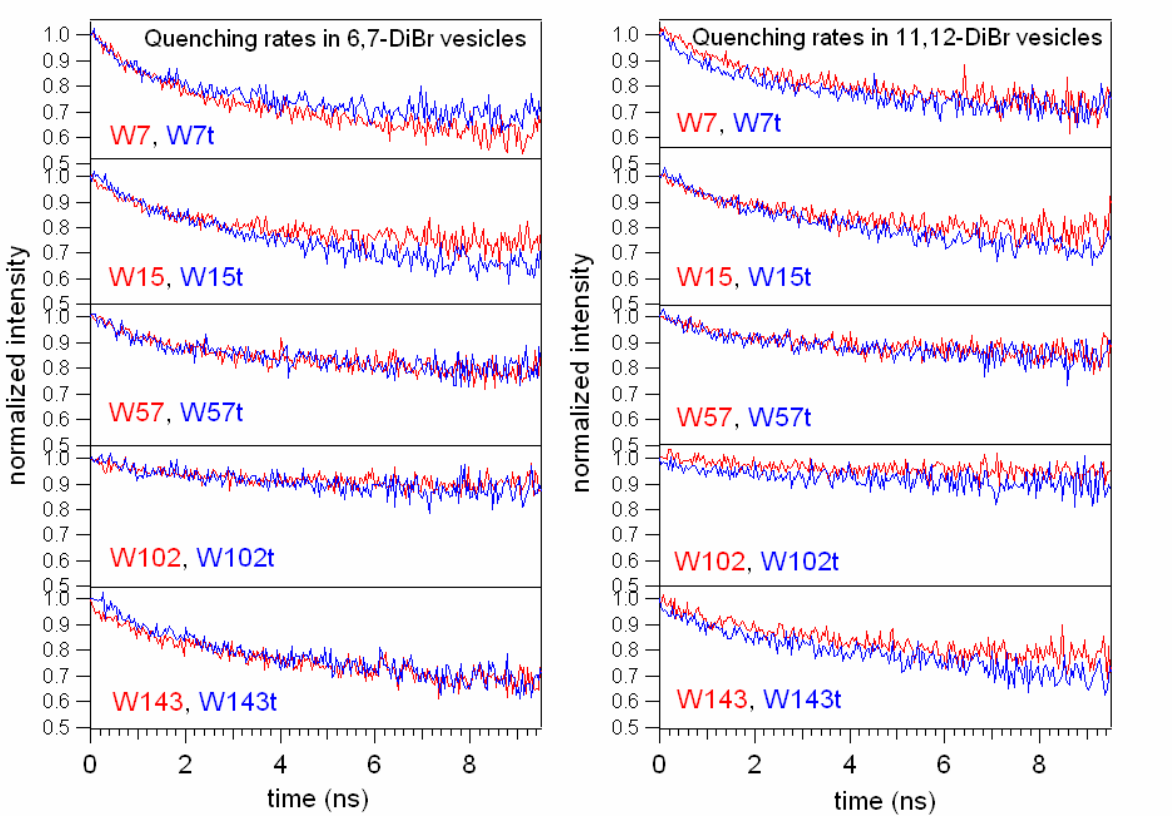


Figure 4.9. Overlay of the quenching rates of full-length mutants (red) and truncated mutants (blue) folded in 6,7-DiBr (left) and 11,12-DiBr (right).

Mutant	F/F_o 6,7-DiBr	F/F_o 11,12-DiBr	Quenching rates (ns) 6,7-DiBr	Unquenched fraction 6,7-DiBr	Quenching rates (ns) 11,12-DiBr	Unquenched fraction 11,12-DiBr
W7	0.56	0.76	2.3	0.51	2.6	0.67
W7t	0.62	0.77	1.4	0.65	1.3	0.71
W15	0.64	0.77	1.8	0.72	0.81	0.74
W15t	0.63	0.77	3.1	0.58	0.83	0.64
W57	0.71	0.88	2.2	0.71	0.75	0.83
W57t	0.59	0.71	1.4	0.78	0.72	0.83
W102	0.67	0.74	2.3	0.90	0.87	0.95
W102t	0.82	0.91	3.1	0.86	0.68	0.89
W143	0.67	0.80	2.9	0.58	0.76	0.76
W143t	0.63	0.73	2.9	0.58	0.81	0.61

Table 4.1. Ratios of quenched (F) to unquenched (Fo) emission maxima for 6,7-DiBr and 11,12-DiBr. The quenching rates (ratio of quenched to unquenched fluorescence decays) shown are amplitude weighted average lifetimes from biexponential fits ($y = c_o + ae^{-k1t} + be^{-k2t}$). The unquenched fractions (c_o) are also listed.

4.4 CONCLUSIONS

Vesicles composed of brominated lipids were used to investigate the fluorescence quenching of specific Trps in full-length and truncated mutants in the folded state at 30^{o}C. The aim was to identify whether Trp residues in the truncated mutants displayed the same degree of quenching as the Trp residues in the full-length mutants. While steady-state fluorescence data was ambiguous, time-resolved fluorescence confirmed that W7 is the most quenched and W102 is the least quenched. This is consistent with previous reports and the high-resolution structure of OmpA that shows the pore-facing orientation of W102. W7 appears to be the closest to the hydrocarbon center compared to the other Trp residues. Although the use of brominated lipids was useful for studying Trp locations in relation to the bilayer, new insight into the OmpA transmembrane structure was not revealed since the truncated and full-length Trp locations are similar. This is consistent with our fluorescence data in Chapter 3 that showed the transmembrane environment was minimally affected by the C-terminal domain. While new information was not gained from these experiments, the fluorescence quenching techniques used both in steady-state and time-resolved fluorescence would be useful for studying less characterized integral membrane proteins to reveal new insights into the structure.

4.5 REFERENCES

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