STRUCTURAL DETERMINATION AND TRYPTOPHAN FLUORESCENCE OF HETEROKARYON INCOMPATIBILITY C2 PROTEIN (HET-C2), A FUNGAL GLYCOLIPID TRANSFER PROTEIN (GLTP), PROVIDE NOVEL INSIGHTS INTO GLYCOLIPID SPECIFICITY AND MEMBRANE INTERACTION BY THE GLTP-FOLD*

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Running Title: HET-C2 Glycolipid Specificity and Membrane Interaction

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Supplementary Information

Glycolipid intervesicular transfer activity. In the radiolabeled assay [*30,32*], donor membrane vesicles prepared by sonication and composed of 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC), negatively-charged phospholipid (10 mol%), [^{14}C]-glycolipid (2 mol%), and a trace of [^{3}H]-tripalmitin (nonexchangeable marker) were incubated at 37°C with HET-C2 (0.1-1.0 μg) and tenfold excess of neutral POPC acceptor vesicles (4 mM concentration). Glycolipid transfer was quantified by liquid scintillation counting of acceptor vesicles recovered after elution through DEAE minicolumns. The fluorescence-based resonance energy transfer (RET) assay involved anthrylvinyl(AV)-labeled glycolipid (1 mol%) and nontransferable perylenoyl-labeled PC (1mol%) and permitted continuous real time monitoring of GLTP activity [*31,33*]. Excitation and emission wavelengths were 370 nm and 425 nm, respectively. POPC donor vesicles containing the fluorescent lipids were prepared by rapid ethanol injection; POPC acceptor vesicles, by sonication. Initial transfer velocities and other details of this established assay are presented elsewhere [*31,33*]. Calculation of glycolipid transfer rates to acceptor vesicles were analyzed by fitting to first order exponential behavior using OriginPro 7.5 (Northampton, MA). Radiolabeled GlcCer, GalCer, and sulfatide were obtained from American Radiochemical. Radiolabeling and purification of LacCer and ganglioside GM1 were accomplished as described previously [*66*].

Preparation of small unilamellar vesicles. SUV bilayer vesicles were prepared by sonication. Briefly, a lipid film was obtained by rotary evaporation of the desired lipid mixture in solvents, followed by 6h of drying *in vacuo*. A lipid suspension was produced by vortexing in sodium phosphate buffer (pH 7.4). Probe sonication under nitrogen and on ice was performed with a Heat Systems-Ultrasonics W-225 sonifier until translucent [*67*]. Centrifugation at 100,000g for 90 min pelleted titanium probe particles and residual multilamellar vesicles. Analysis by size exclusion chromatography [*68*] confirmed average vesicle diameters of ~25-30 nm.

CD Secondary Structure Calculations. Two sets of reference proteins were used for secondary structure calculation: 48 proteins [*29*,*34*], or 23 α+β reference proteins, selected by the CLUSTER program. The average value of the six quantities (three methods and two reference sets) ±RMS deviations are presented in Table 1. Secondary structure was classified according to [*35*], using the DSSP program [*29*]. DSSP output included α-helix (H), 3_{10}-helix (G), *β*-sheet (E), *β*-bridges (B), turns (T), and bends (S), which were subgrouped as follows: the α- and 3_{10}-helix were treated in CDPro as helix; *β*-sheet as *β*-strand; turns and

bends were treated as turns; a minimum of two adjacent residues were required for such grouping for turns and bends. Single residues assigned to a structure (such as β-bridges, turns, and bends) were grouped as unordered, which contains residues that are not assigned to any defined structural class. The number and average length of the secondary structure segments also were assessed using CDPro. The number of secondary structure segments was calculated by dividing the number of residues included in the distorted helical structure by factor of four and, in the distorted β structure, by factor of two. Dividing the total residues in the α helix or β strand (ordered plus distorted) by the number of segments gave the average segment length. Tertiary structure class was determined by the CLUSTER program [*37*] of CDPro.

Trp Fluorescence Quenching Analysis. Data collected in the presence of acrylamide and KI were analyzed using the Stern-Volmer equation:

$$F_o/F = 1 + K_{SV}[Q] \quad (1)$$

where F_o and F are fluorescence intensities in the absence and presence of quencher; [Q] is quencher concentration; and K_{SV} is the Stern-Volmer quenching constant [*45,56,64,65*]. The modified Stern-Volmer equation (Lehrer plot) was used to calculate the fraction of total intensity accessible to quencher.

$$F_o/\Delta F = 1/(f_a K_Q[Q]) + 1/f_a \quad (2)$$

where F_o and [Q] are same as defined earlier and ΔF is the change in the fluorescence intensity due to quenching, K_Q is the modified Stern-Volmer quenching constant and f_a is the fraction of the initial fluorescence which is accessible to the quencher [*45,56,69,70*].

Supplemental References

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Table S1. Far-UV CD secondary structural determinations for HET-C2, HET-C2 + POPC , and HET-C2 + (POPC / *N*-octanoyl-LacCer)

		HET-C2	HET-C2 + POPC	HET-C2 + (POPC /LacCer)	Average± RMSD
Helix	*regular*	34.9	33.0	33.3	33.7 ± 1.0
	distorted	21.6	21.5	21.2	21.4 ± 0.2
	total	56.6	54.5	54.5	55.1 ± 1.2
β–strand	*regular*	4.2	4.8	4.3	4.4 ± 0.3
	distorted	4.6	4.7	4.5	4.6 ± 0.1
	total	8.8	9.5	8.8	9.0 ± 0.4
Turns		12.1	12.6	13.0	12.5 ± 0.5
Unordered		24.1	23.9	24.2	24.0 ± 0.2
Number of helical segments		11.2	11.1	11.0	11.1 ± 0.1
Average length of helix		10.6	10.2	10.3	10.4 ± 0.2
Number of *β*–strands		4.7	5.0	4.7	4.8 ± 0.2
Average length of *β*–strand		3.8	4.0	3.9	3.9 ± 0.1
Tertiary structure class		α + *β*	α + *β*	α + *β*	α + *β*

* *regular helix* or *β-strand*: fraction of residues (%) in central part of helical segments or strands;
distorted helix or *β-strand*: fraction of terminus residues (%) in helices (two at each end of helix, totally four per helical segment) or *β*-strands (one residue at each end of strand, totally two per strand);
total: *regular* + *distorted*;
average length of helix or *β-strand*: average number of residues per helix or *β*-strand, a translation per one residue is equal to 1.5 Å for α-helix and 3.3 Å for *β*-strand.

Secondary structure calculations were carried out as described in the Experimental Procedures and Supplementary Information. The far-UV CD data analyses for apo-HET-C2 indicated 56.6% helix, consisting of 11 segments, each averaging 10 residues; 8.8% β-structure, consisting of five strands, each averaging four residues; 12.1% β-turns, and 24.1% random. The values agree well with similar calculations for apo-GLTP (PBD 1SWX). Incubation of HET-C2 with POPC vesicles either lacking or containing glycolipid resulted in little change in the far-UV CD spectra. Secondary structure calculations indicated 54.5% α-helix, 9% β-structure, 12.8% β-turns, and 24% random.

Figure S1

Di-methylated lysine residues present in HET-C2 structure. 13 out of 18 lysine residues present in HET-C2 showed proper density for the di-methyl group. The side chain atoms of these residues are colored in magenta.

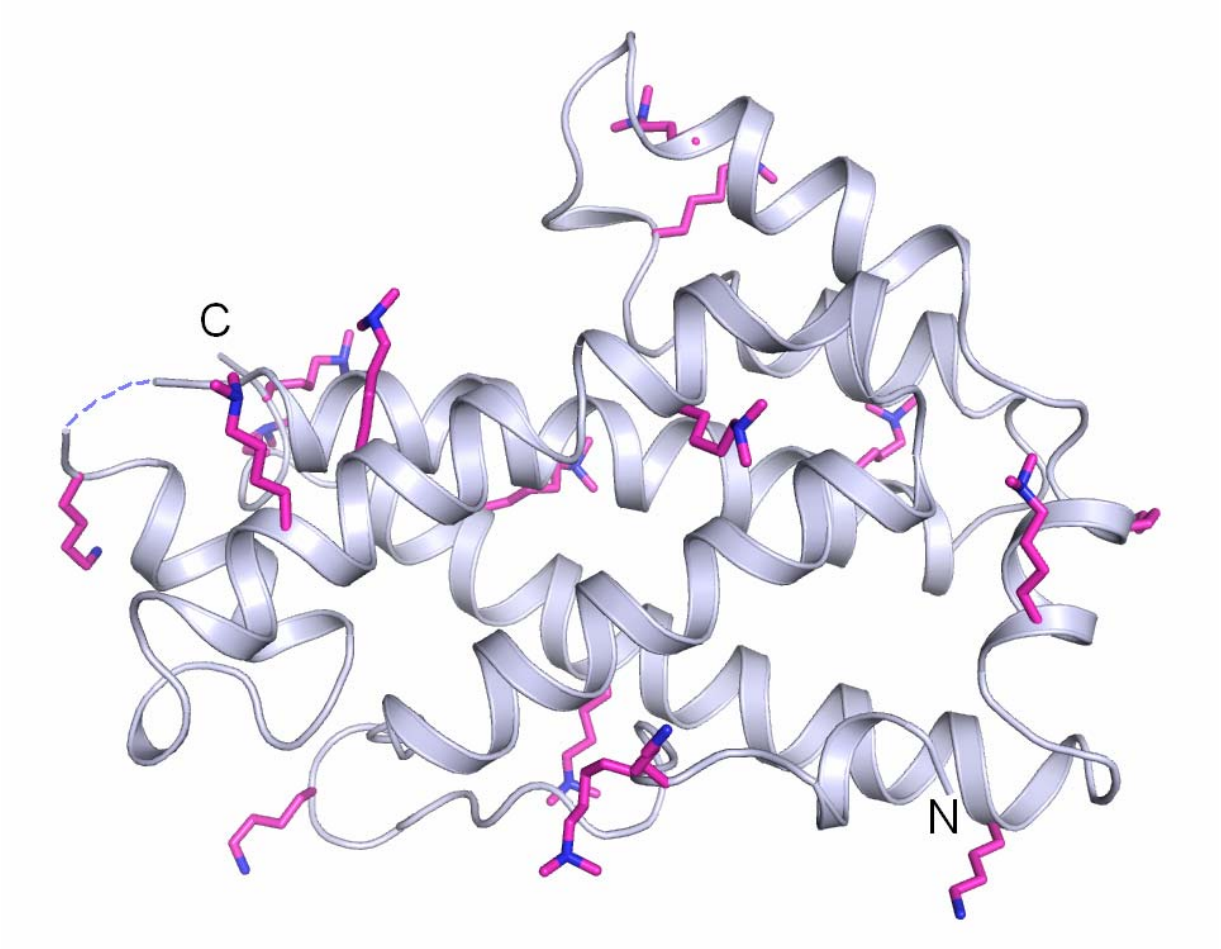

Figure S2

Electrostatic surface representation of HET-C2 (left) and GLTP (right), highlighting the key residues involved in binding of the glycolipid head group. Positively charged residues are shown in blue, negatively charged residues are shown in red, and neutral residues are shown in white.

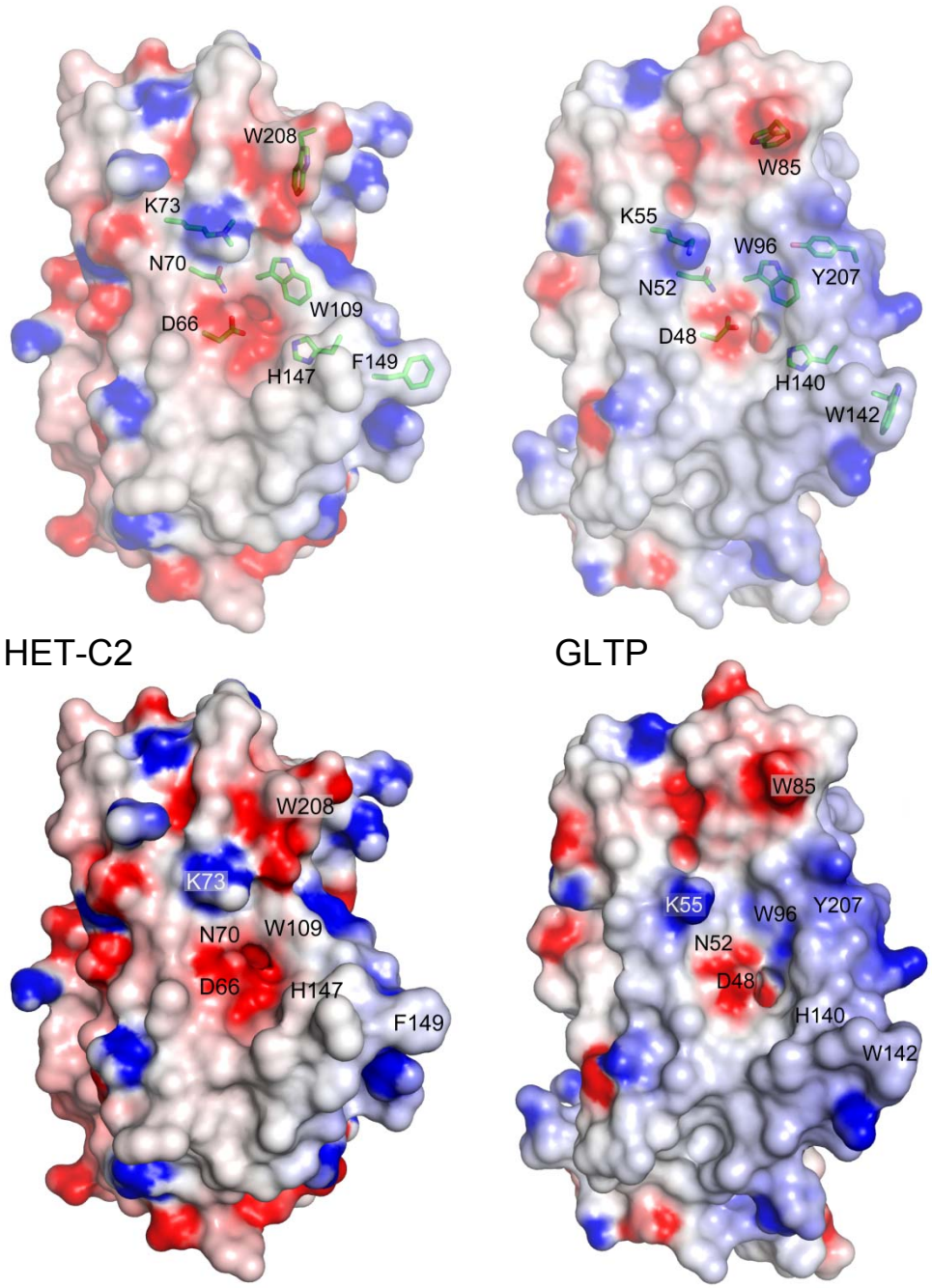

Figure S3: Trp Emission of apoHET-C2 in phosphate buffered saline (PBS) at pH 7.4, in 8 M urea plus PBS, and in presence of POPC vesicles containing 20 mol% glycolipid plus PBS. The Trp emission λ_{max} for apoHET-C2 is ~355 nm, is further red-shifted (~2 nm) by denaturation in urea, but is blue-shifted (~6 nm) upon incubation with membranes containing glycolipid. Protein concentration was 1 μM and excitation = 295 nm. Fluorescence emission measurements were performed as detailed in the Exptl. Proc.

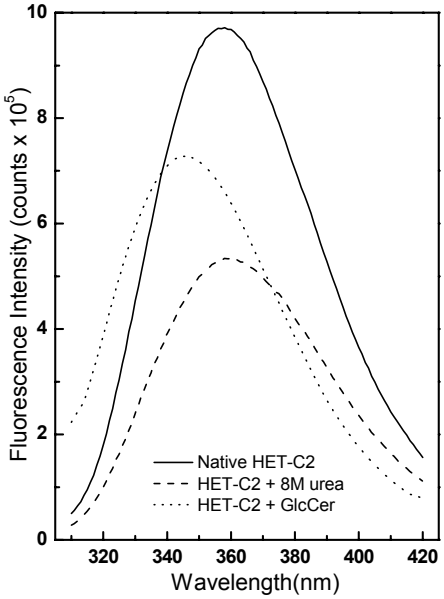

Figure S4: Fluorescence quenching of Trp emission with increasing concentrations of acrylamide. The spectra shown are: **(A)** wt HET-C2; **(B)** HET-C2 mixed with POPC:GalCer (8:2) SUVs; **(C)** HET-C2 denatured with 8M Urea. Protein samples in PBS were titrated with small aliquots of 5M stock quencher solution (final acrylamide concentrations of 0.01, 0.02, 0.04, 0.06, 0.09, 0.12, 0.16, 0.19, 0.24, 0.28, .33, 0.37M) and fluorescence spectra were recorded after each addition. The vertical arrows indicate increasing quencher concentration. In (B), protein was preincubated for 30 min in the presence of POPC:GalCer (8:2) SUV before adding any quencher. The percentage of maximum quenching achieved at a quencher concentration of 0.37M is shown in Table 2.

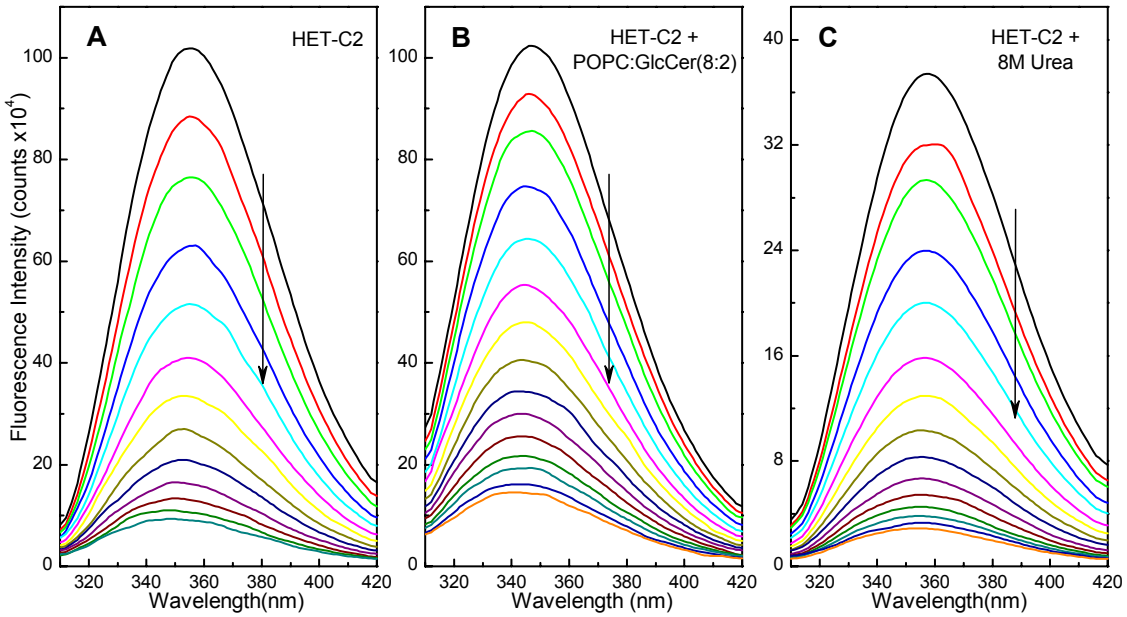

Figure S5: Modified SV plots of acrylamide quenching of wt HET-C2 in solution or mixed with POPC vesicles containing or lacking glycolipid. From the *Y*-intercepts of these plots, f_a , the value of the fraction of accessible Trp residues was determined for each case and the value of the corresponding quenching constant, K_a , was calculated using Eq. (2), described above in the Supplementary Info. These values are also listed in Table 2.

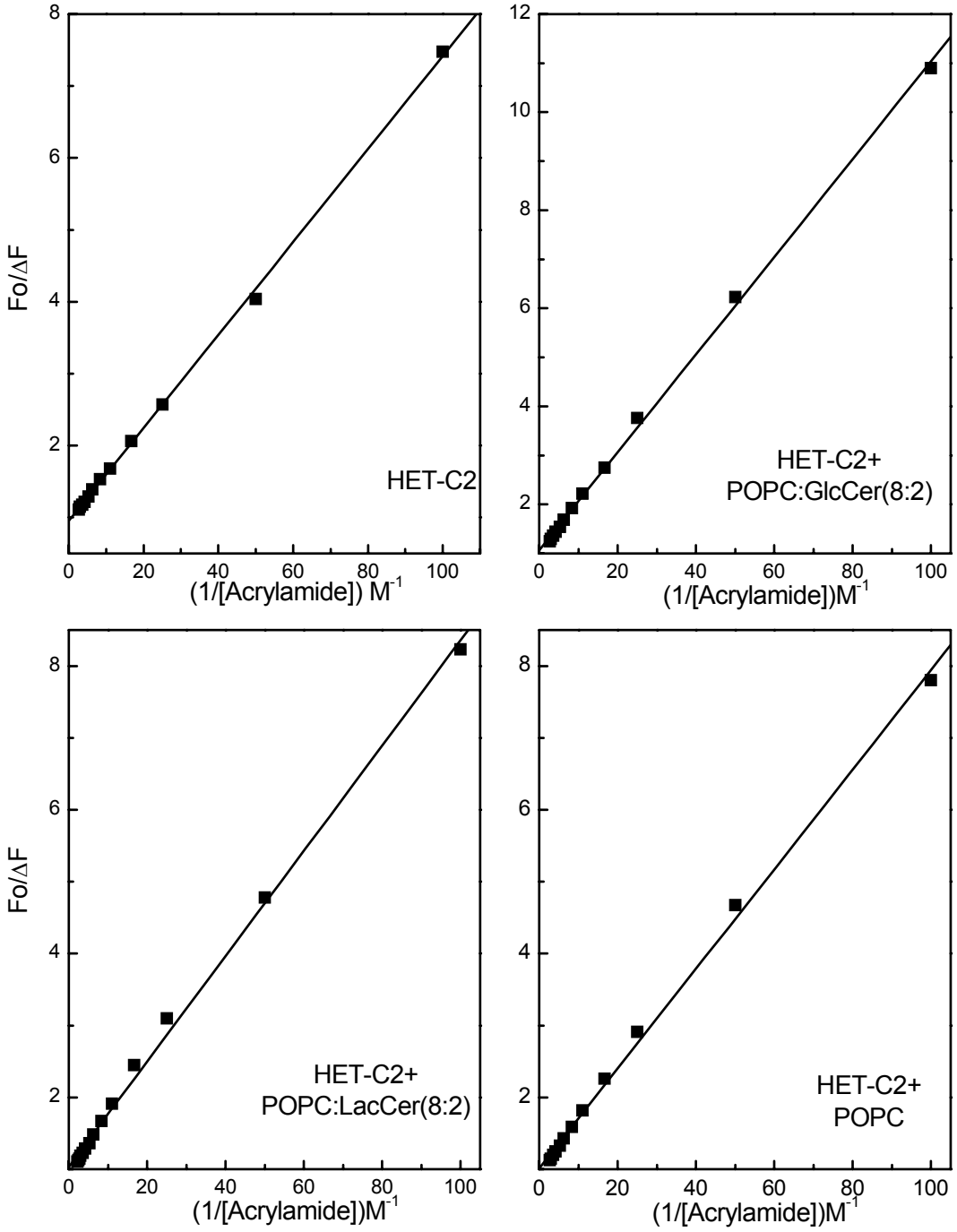

Figure S6: Fluorescence quenching of HET-C2 with increasing concentrations of KI along with the corresponding Stern Volmer (SV) and modified SV plots. Protein samples in PBS were titrated with small aliquots of 5M stock quencher solution (final KI concentrations of 0.01, 0.02, 0.04, 0.06, 0.09, 0.12, 0.16, 0.19, 0.24, 0.28, .33, 0.37, 0.42M) and fluorescence spectra were recorded after each addition. KI stock solution was stabilized with 10 mM sodium thiosulfate to prevent the formation of I_3^-, which absorbs in the region of Trp fluorescence. The vertical arrow in A indicates increasing quencher concentration. The quenching data are summarized in Table 2.

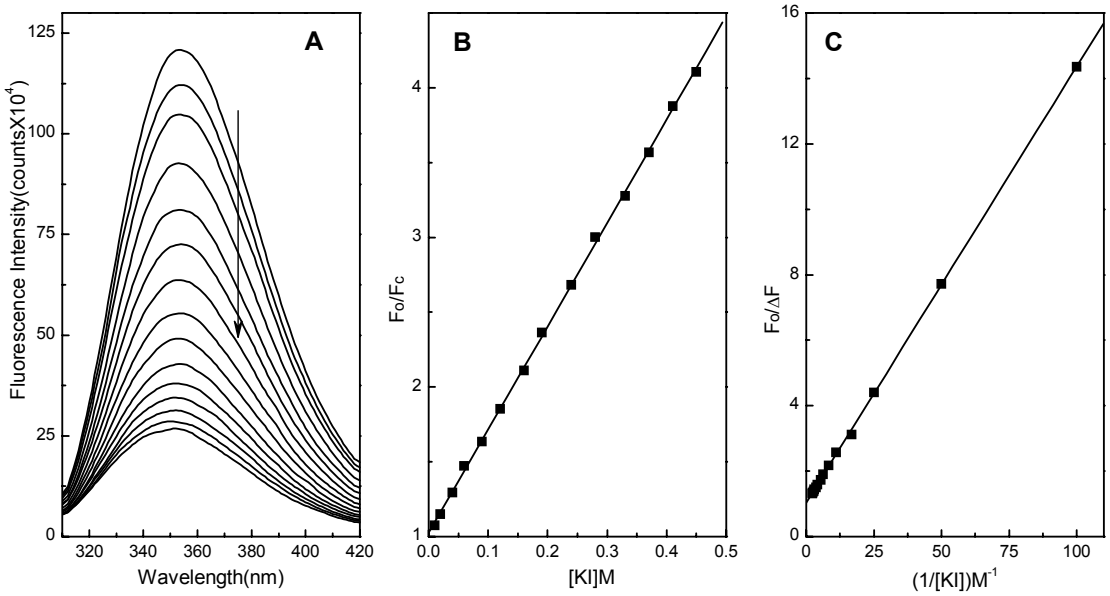

Figure S7:

Surface Hydrophobicity of HET-C2 (PDB 3KV0). Mapping was performed using Chimera [*58,59*], which relies on the Kyte-Doolittle scale to rank amino acid hydrophobicity, with blue indicating most hydrophilic, white equaling 0.0, and orange-red being most hydrophobic. The pit-like morphology surrounding W109 is apparent as well as the large hydrophobic patch (F149, L150, L121, A157, A161, A59, I58, F60) predicted to reflect the membrane docking area.