Using Two Fluorescent Probes to Dissect the Binding, Insertion, and Dimerization Kinetics of a Model Membrane Peptide

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Peptide Sample Preparation: Antiα_{IIb}-Phe$_{CN}$ and anti-α_{IIb} peptides were synthesized using standard Fmoc-based solid-phase peptide synthesis protocols, purified by reverse-phase HPLC, and verified by electrospray ionization mass spectrometry. The peptide solutions were prepared by first dissolving lyophilized peptide in a small amount of ethanol, which was then diluted with either 25 mM HEPES buffer (pH 7.0) or 25 mM Tris buffer (pH 7.0, for CD measurements). The final peptide solution contains 5% (v/v) ethanol, and the peptide concentration was determined optically using the sample absorbance at 280 nm along with a total molar extinction coefficient calculated based on those of Trp (5600 M^{-1} cm^{-1}), Tyr (1200 M^{-1} cm^{-1}), Phe (72.5 M^{-1} cm^{-1}), and Phe$_{CN}$ (855 M^{-1} cm^{-1}).

Vesicle Preparation: A mixture of POPC/POPG (3:1, wt/ wt) with a total lipid concentration of 1.72 mg/ml was first prepared in 25 mM HEPES or Tris (for CD measurements) buffer (pH 7.0) containing 5% ethanol. This mixture was then taken through a freeze-thaw-vortex process. After this process was repeated five times, the vesicle suspension was extruded through a polycarbonate membrane filter with pore diameters of 200 nm or 50 nm (for CD measurements) by an extruder (Avanti Polar Lipids).

CD and Fluorescence Measurements: The CD spectra were collected at 20 ^{o}C on an AVIV 62DS spectropolarimeter (Aviv Associates, NJ) using a 1 cm quartz cell. The fluorescence spectra were measured at 20 ^{o}C on a Fluorolog 3.10 spectrofluorometer (Jobin Yvon Horiba, NJ) using a 1 cm quartz sample holder and 2 nm spectral resolution (excitation and emission).

Stopped-flow Measurements: The stopped-flow fluorescence kinetics were measured at 20 ^{o}C using a SFM-300 stopped-flow module (Bio-logic, Claix, France) equipped with home-built optics, the details of which have been described in Tucker *et al. J. Phys. Chem. B* **2006**, *110*, 8105-8109. The flow rate used in the current study was 10 mL/s, which resulted in a dead time of about 1 ms. Peptide-vesicle association reactions were initiated by mixing equal volumes of the desired peptide and vesicle solutions. For experiments where Trp fluorescence was excited at 290 nm, a 315 nm long-pass filter was used to eliminate the excitation light, whereas for experiments where Phe$_{CN}$ fluorescence was excited at 240 nm, a home-built DMSO filter was used to eliminate >99.5% of the excitation light. Fitting the stopped-flow kinetics to the kinetic model discussed in the text was achieved using the program Scientist (Micromath Research, LC). The fitting parameters include all microscopic rate constants ($k_{\pm i}$), the fluorescence intensities of all species, and the percentage of monomeric peptide (P_m%) in solution. In addition, the total peptide concentration was also treated as a fitting parameter. However, it was found that the recovered peptide concentration from the fitting was within the range of ±10% of the experimentally determined value.

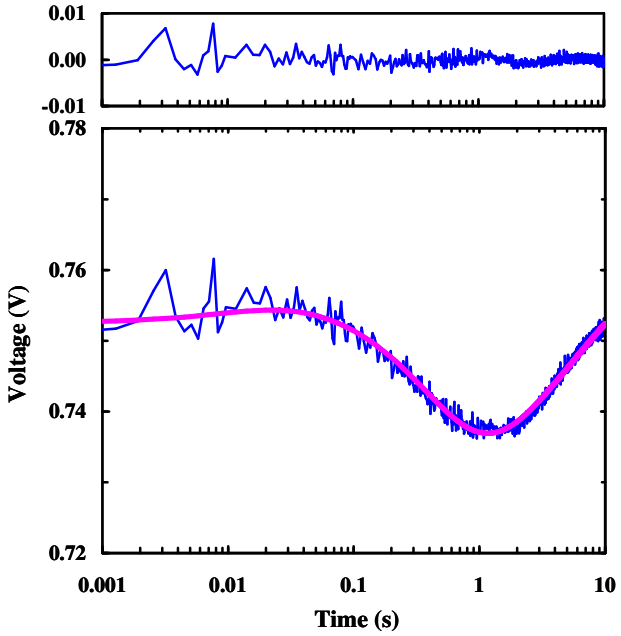

Figure S1. Stopped-flow kinetics of anti-α_{IIb}-Phe_{CN} upon interaction with POPC/G vesicles (wt/wt, 3/1, 0.86 mg/mL). The final peptide concentration was 1.0 μM. The smooth line represents the best fit of these data to the kinetic model discussed in the text, and the fitting parameters are listed in Table S1 and S2. Also shown (top panel) are the residuals of the fit.

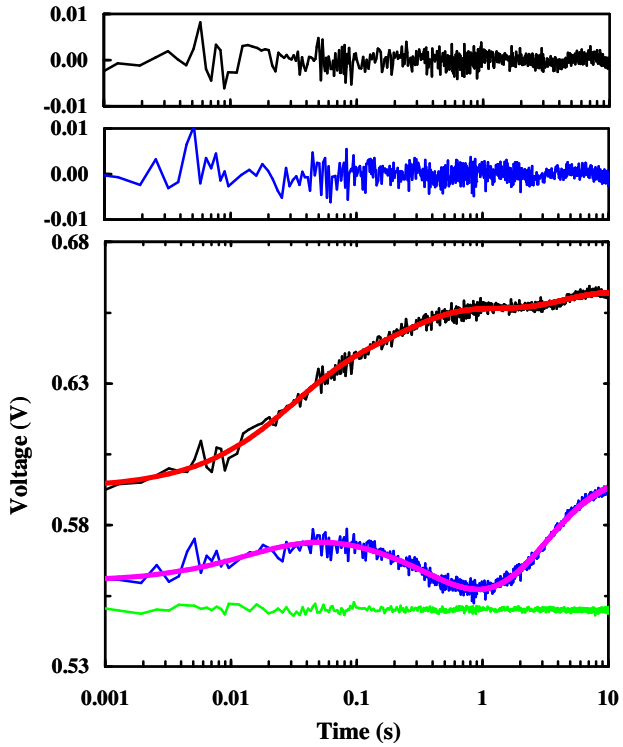

Figure S2. Stopped-flow kinetics of anti-α_{IIb}-Phe_{CN} (blue) and anti-α_{IIb} (black) upon interaction with POPC/G vesicles (wt/wt, 3/1, 0.86 mg/mL). In both cases the final peptide concentration was 2.5 μM. The smooth lines represent the best fits of these data to the kinetic model discussed in the text, and the fitting parameters are listed in Table S1 and S2. Also shown (top panels) are the residuals of these fits, as well as stooped-flow kinetics (green) obtained from control experiments wherein a 5 μM anti-α_{IIb}-Phe_{CN} solution was diluted to 2.5 μM by buffer. All anti-α_{IIb}-Phe_{CN} data have been offset for comparison.

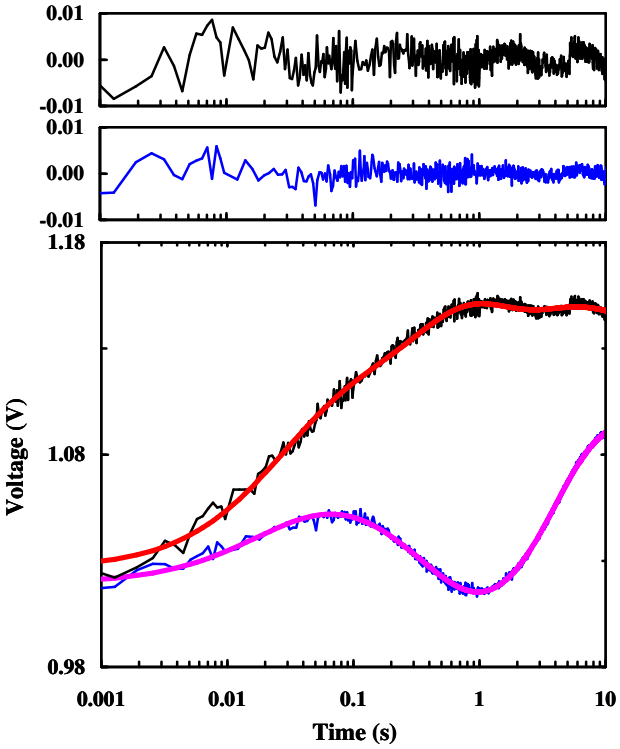

Figure S3. Stopped-flow kinetics of anti-α_{IIb}-Phe_{CN} (blue) and anti-α_{IIb} (black) upon interaction with POPC/G vesicles (wt/wt, 3/1, 0.86 mg/mL). In both cases the final peptide concentration was 5.0 μM. The smooth lines represent the best fits of these data to the kinetic model discussed in the text, and the fitting parameters are listed in Table S1 and S2. Also shown (top panels) are the residuals of these fits. The anti-α_{IIb}-Phe_{CN} data have been offset for easy comparison.

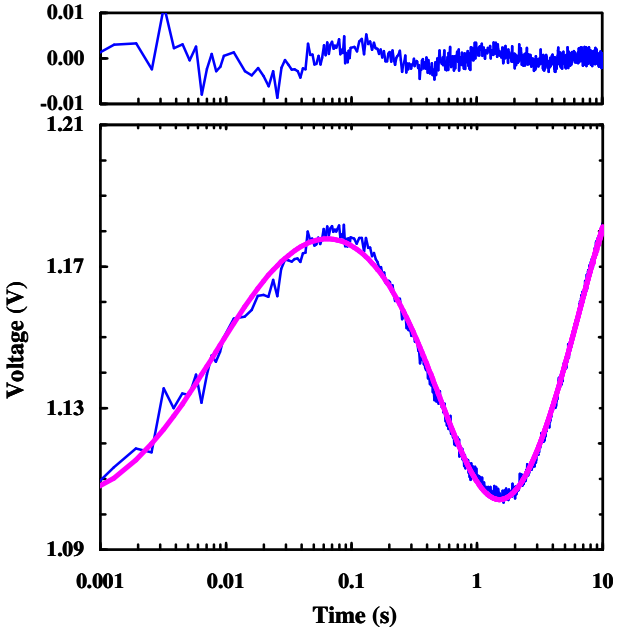

Figure S4. Stopped-flow kinetics of anti-α_{IIb}-Phe_{CN} (blue) upon interaction with POPC/G vesicles (wt/wt, 3/1, 0.86 mg/mL). The final peptide concentration was 15 μM. The smooth line represents the best fit of these data to the kinetic model discussed in the text, and the fitting parameters are listed in Table S1 and S2. Also shown (top panel) are the residuals of the fit.

Table S1. Rate constants recovered from fittings discussed in the text. All second order rate constants were determined with respect to the peptide concentration in solution. It is worth pointing out that the value of the bimolecular association rate constant, k_3, would be different than that reported here if it is expressed with respect to the 'concentration' of the membrane-bound peptide or peptide/lipid ratio.

		k_1 (M^{-1} s^{-1})	k_2 (s^{-1})	k_3 (M^{-1} s^{-1})	k_4 (M^{-1} s^{-1})	k_5 (s^{-1})
Anti-α_{IIb}-Phe_{CN}	1.0 μM	$(7.0 \pm 0.9) \times 10^6$	2.3 ± 0.1	$(1.5 \pm 0.1) \times 10^5$	$(9.0 \pm 0.9) \times 10^4$	0.50 ± 0.04
	2.5 μM	$(5.9 \pm 0.7) \times 10^6$	2.0 ± 1.3	$(1.5 \pm 0.3) \times 10^5$	$(9.9 \pm 0.6) \times 10^4$	0.57 ± 0.07
	5.0 μM	$(4.5 \pm 1.8) \times 10^6$	2.2 ± 0.1	$(1.8 \pm 0.2) \times 10^5$	$(1.1 \pm 0.2) \times 10^5$	0.43 ± 0.02
	15 μM	$(9.6 \pm 0.5) \times 10^6$	1.1 ± 0.1	$(1.5 \pm 0.1) \times 10^5$	$(1.5 \pm 0.1) \times 10^5$	0.30 ± 0.01
Anti-α_{IIb}	2.5 μM	$(3.4 \pm 0.3) \times 10^6$	3.9 ± 0.9	$(1.1 \pm 0.4) \times 10^5$	$(0.7 \pm 0.3) \times 10^5$	0.52 ± 0.14
	5.0 μM	$(4.0 \pm 0.5) \times 10^6$	2.6 ± 0.6	$(1.3 \pm 0.3) \times 10^5$	$(1.0 \pm 0.4) \times 10^5$	0.60 ± 0.04

Table S2. Relative fluorescence intensities (F) and the percentage of peptide monomer in solution, P_m %, recovered from the fittings discussed in the text.

		F(P_m)	F(P_m^s)	F(P_m^i)	F(D)	F(P_d)	F(P_d^s)	P_m %
Anti-α_{IIb}-Phe_{CN}	1.0 μM	1.0	1.02±0.01	0.89±0.01	2.14±0.01	2.02±0.1	0.06±0.01	99 ± 1
	2.5 μM	1.0	1.05±0.01	0.99±0.02	2.24±0.01	2.13±0.2	0.08±0.01	94 ± 3
	5.0 μM	1.0	1.09±0.01	1.01±0.01	2.27±0.02	1.89±0.06	0.10±0.01	85 ± 1
	15 μM	1.0	1.14±0.01	0.96±0.01	2.23±0.01	1.76±0.02	0.14±0.01	70 ± 2
Anti-α_{IIb}	2.5 μM	1.0	1.09±0.02	1.14±0.01	2.26±0.01	1.99±0.06	1.54±0.12	93 ± 1
	5.0 μM	1.0	1.11±0.03	1.19±0.05	2.22±0.09	1.76±0.13	1.53±0.15	86 ± 1

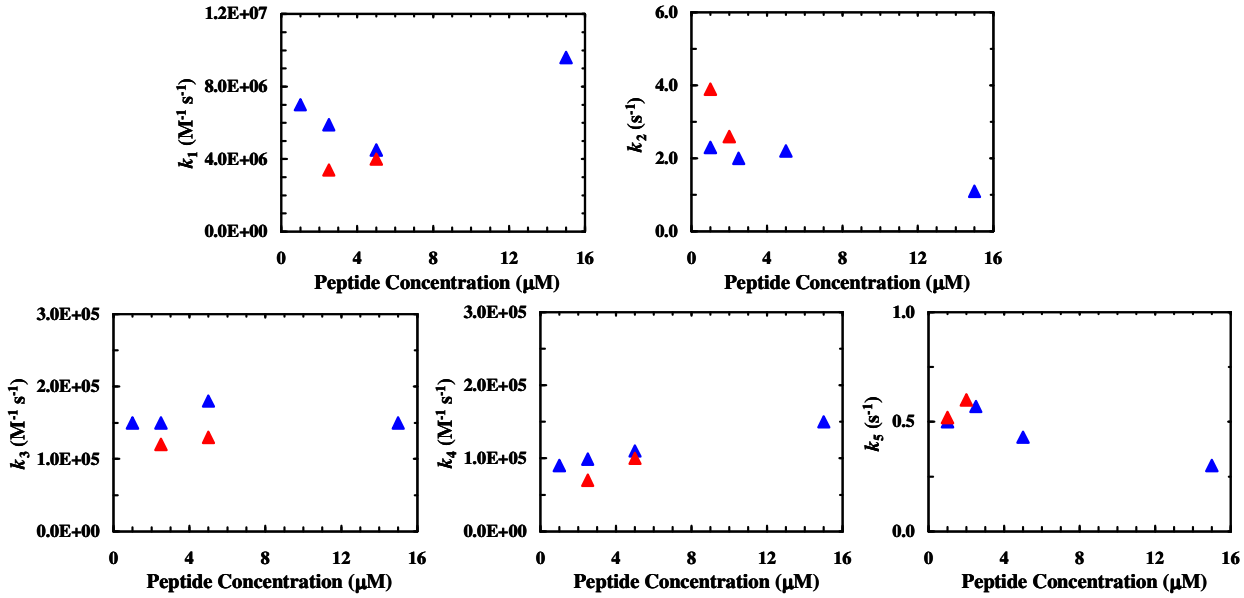

Figure S5. Comparison of the rate constant values (in Table S1) obtained at different peptide concentrations (blue - anti-α_{IIb}-Phe_{CN}, red - anti-α_{IIb}).