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Supporting Information for:

NMR Characterization of Cooperativity: Fast Ligand Binding Coupled to Slow Protein Dimerization.

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Proteins and Peptide Preparation. MCP-1 was expressed and purified as described by Tan *et al* (*J. Biol. Chem*. 2012, *287*(18), 14692). Briefly, an N-terminal His_6-tagged form of MCP-1 was expressed in *E.coli* using minimal media to allow ^{15}N-enrichment. Inclusion bodies containing the fusion protein were isolated and dissolved in denaturing buffer and then purified by Ni^{2+}-affinity chromatography. The fusion protein was refolded by drop wise dilution into native buffer, the His_6-tag was removed using human α-thrombin and the protein was further purified by cation exchange chromatography. The CCR2 sulfopeptides **1** and **2** were prepared by solid-phase synthesis and purified as described (Taleski *et al, Chem. Asian J*. 2011 *6*, 1316-1320).

NMR Measurements. Samples for NMR spectroscopy (150 μL in Norell thick-walled 5 mm NMR tubes) contained 50μM wild type MCP-1 alone or in the presence of 10, 20, 35, 50, 80 or 150 μM peptide (**1** or **2**) in NMR buffer (20 mM sodium acetate-d_4, 5% D_2O, 0.02% NaN_3, pH 7.0). NMR experiments were conducted at 25 °C on a Bruker Avance 600 MHz NMR spectrometer equipped with a triple-resonance cryoprobe. Chemical shifts were referenced to 4, 4-dimethyl-4-silapentane-1-sulfonic acid (DSS). For each sample a ^{15}N-heteronuclear single quantum coherence (HSQC) spectrum was recorded using 48 and 1024 complex points and spectral widths of 24 and 12 ppm in ^{15}N and ^{1}H dimensions, respectively, and 276 scans per FID (experiment time: 8hrs 45 min). The NMR data were processed using Bruker Topspin 3.0.

NMR Spectra Analysis. NMR spectra were analyzed using Sparky (T. D. Goddard and D. G. Kneller, University of California, San Francisco, CA). Weighted changes in NH chemical shift for resolved monomer and dimer peaks (*m* and *d*, respectively) were determined according to the formula: $\Delta\delta_{NH}$

$= |\Delta\delta_H| + 0.2|\Delta\delta_N|$. The standard errors in chemical shift measurements were estimated by analysis of peaks that did not shift monotonically with peptide concentration. Relative intensities of monomer and dimer peaks (r_{MD}) were calculated from the heights of corresponding monomer and dimer peaks; peak volumes could not be determined with sufficient precision or accuracy due to spectral noise and overlap. The standard errors in the peak heights were assumed to be equal to the standard deviation of the base plane noise level. Average values of *m*, *d* and r_{MD} (and their standard errors) were calculated for the five NH groups showing resolving monomer and dimer peaks at all concentrations of peptide used: K19, L25, I42, F43 and C52.

Data Fitting Procedures. Global fitting of the NMR data to the coupled thermodynamic model were performed using a home-written Perl script. Briefly, the script performs a series of increasingly focused grid searches to find values of K_{MD}, K_{ML} and K_{DL} in closest agreement with the observed *m*, *d* and r_{MD} values (each at several concentrations of ligand); typically K_{MD} was held constant at the value corresponding to the observed value of r_{MD} in the absence of ligand (K_{MD} = 7.0 μM). For each set of K_{MD}, K_{ML} and K_{DL} values, the simulation algorithm shown schematically in Figure S1 was used to simulate the values of m/m_{max}, d/d_{max} and r_{MD} at each ligand concentration used and then grid searches were used to determine the best fit values of m_{max} and d_{max}. The agreement between the resulting simulated values of *m*, *d* and r_{MD} and the corresponding experimental values was evaluated using the target function χ^2, defined as:

$$\chi^2 = \sum_{L_t}\left[\left(\frac{m_{sim} - m_{expt}}{m_{expt}}\right)^2 + \left(\frac{d_{sim} - d_{expt}}{d_{expt}}\right)^2 + \left(\frac{r_{MD,sim} - r_{MD,expt}}{r_{MD,expt}}\right)^2\right]$$

in which the subscripts "sim" and "expt" refer to simulated and experimentally determined values, respectively, and the sum extends over all values of total ligand concentration (L_t) used in the experiments. The precision of the resulting fitted values of K_{MD}, K_{ML} and K_{DL} was determined using Monte-Carlo (M-C) simulations. For each M-C simulation, input values of *m*, *d* and r_{MD} at each value of L_t were generated randomly accordingly to a Gaussian distribution defined by the average and

standard error of the measured parameter. The fitting procedure was then applied to obtain fitted values of the equilibrium constants. For each data set, 500 M-C simulations were performed, of which the best 95% (475, based on χ^2 values) were used to determine the final values and errors of equilibrium constants and cooperativity. Conventional fits of the *m* and *d* data were performed using the methods described previously (Tan *et al.*, *J. Biol. Chem*. 2012, *287*(18), 14692).

Figure S1. ^{15}N-HSQC Chemical Shifts for Monomeric and Dimeric forms of Residue Cys-52 with peptides 1 and 2. A detailed region (Cys-52 NH resonances) of the ^{15}N-HSQC spectrum is shown for 50 μM MCP-1 alone (red) and in the presence of 20 μM (cyan), 50 μM (orange) and 150 μM (blue) of CCR2 sulfopeptides: (a) **1** and (b) **2**. For each peptide the ratio of monomer to dimer peak intensities (r_{MD}) increases over the titration course. For sulfopeptide **2**, all resonances were substantially weaker in the final titration point compared to the earlier points.

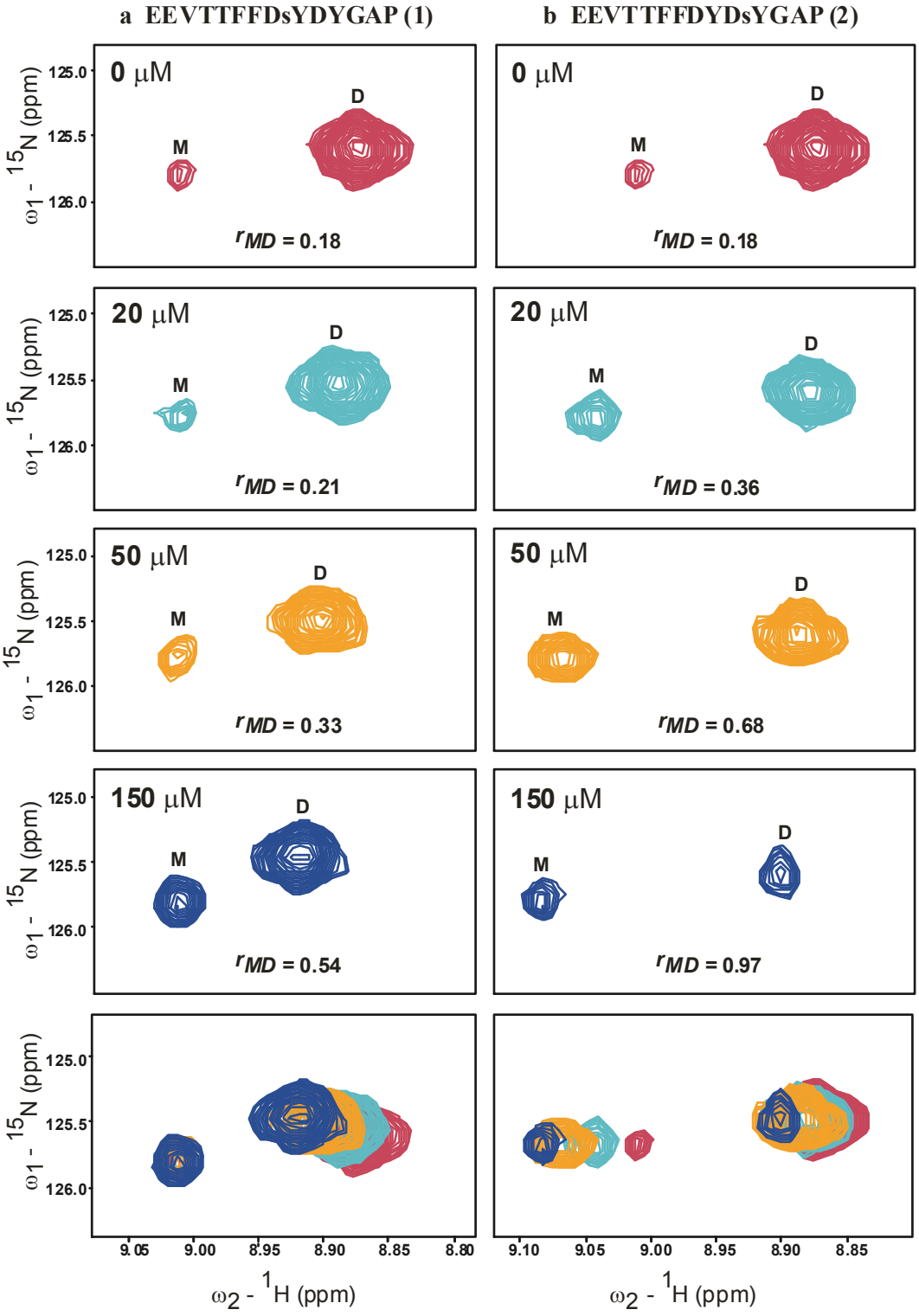

Figure S2. Algorithm for Simulation of Measurable Parameters from Equilibrium Constants in the Coupled Thermodynamic Model. Concentrations of species are adjusted iteratively until the calculated values of equilibrium constants (K_{MD}, K_{ML}, K_{DL} and K_{MDL}) are in close agreement with the input (target) values of equilibrium constants are represented as (*K_{MD}, *K_{ML}, *K_{DL} and *K_{MDL}). Once this condition is satisfied, the species concentrations are used to calculate the values of m/m_{max}, d/d_{max} and r_{MD}.

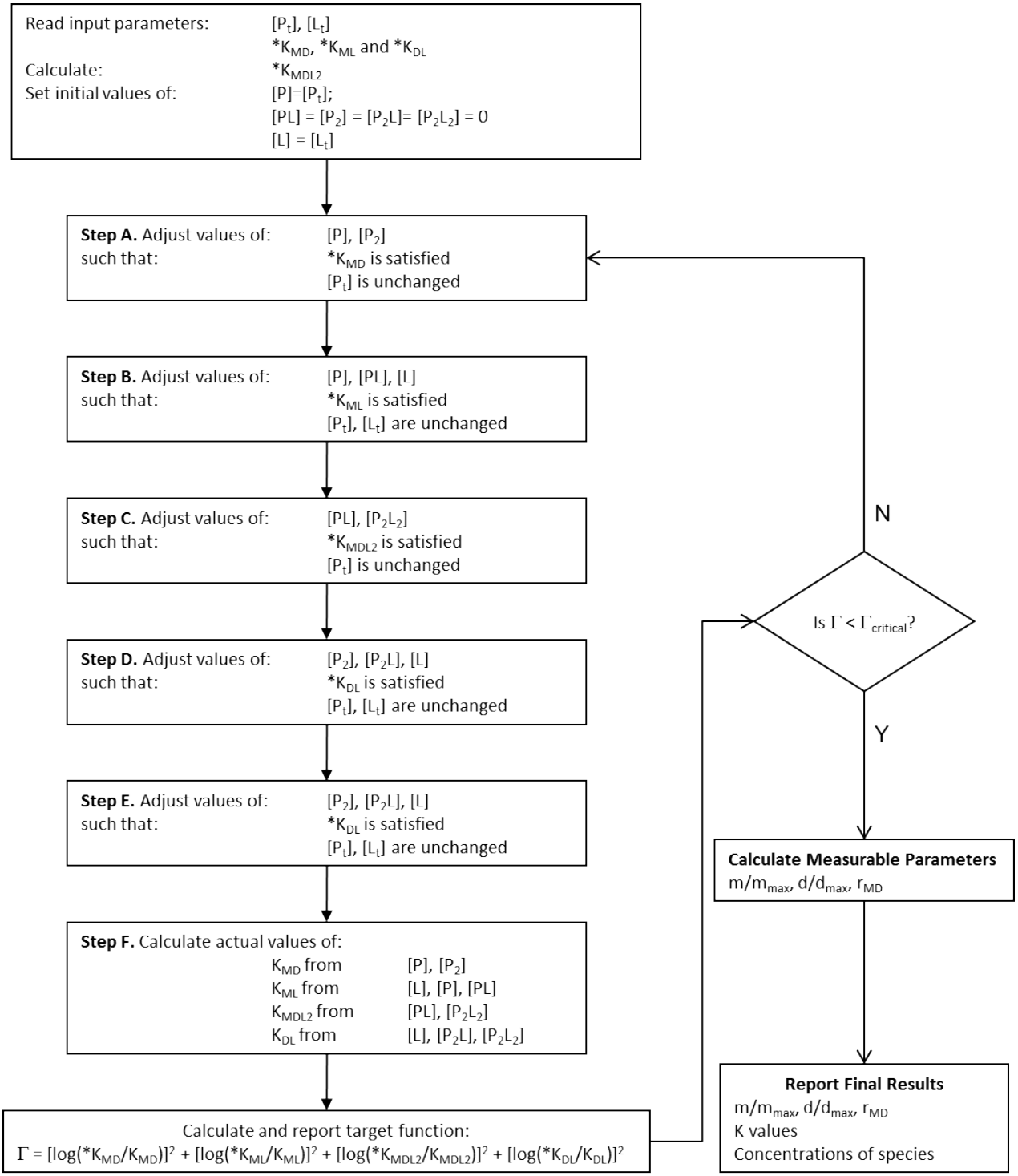

Figure S3. Results of Conventional Fits of WT MCP-1 Monomer and Dimer with Peptides 1 and 2.