

Non-covalent Multivalent Assembly of Jun Peptides on a Leucine Zipper Dendrimer Displaying Fos Peptides

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Supplementary Material

Materials and Methods

General Materials:

All amino acid derivatives, HOBt, BOP and resins used for peptide synthesis were purchased from Novabiochem. All other chemicals and reagents were from Aldrich. HPLC columns were from Vydac.

Peptide Synthesis and Purification:

The peptide-Fos and peptide-Jun were synthesized by solid-phase peptide synthesis using standard Fluorenylmethoxycarbonyl (Fmoc) strategy starting with Rink Amide resin.

Sequences:

PFos: NH₂-CGSGSLTDTLQAETDQLEDEKSALQTEIANLLKEKEKLEFILAA-CONH₂

PJun: NH₂-RIARLEEKVKTTLKAQNSELASTANMLREQVAQLKQKVAASGSGCG-CONH₂

PJun-HC: Coumarin-GGRIARLEEKVKTTLKAQNSELASTANMLREQVAQLKQKVAASGSGCG-CONH₂

The final peptides (PJun) were cleaved from the resin and all protecting groups were removed by the general TFA cleavage (Peptide resin 1g was treated with 10ml cleavage cocktail (81.5% TFA, 5% water, 5% thioanisole, 5% Phenol, 2.5% ethanedithiol and 1% triisopropylsilane) for 90 minutes at room temperature. The solution was filtered and precipitated by transferring into a centrifuge tube containing 50 ml pre-cooled ether. After centrifugation and 3 × washes with fresh ether, the white solid was collected and lyophilized to yield 0.4g crude peptide. The crude peptide was purified by reverse-phase HPLC (Varian) using a C18 column (Vydac). Peptides were purified with a linear gradient of 20% to 80% acetonitrile containing 0.1% TFA at a flow rate of 8 ml/min, for 60 minutes. ~130mg pure peptide was obtained after purification.

Mass Spectroscopy (MALDI) of the two peptides:

The MH⁺ calculated for PFos is 4792.45; found: 4792.63

The MH⁺ calculated for PJun is 4811.61; found 4813.25

The MH⁺ calculated for PJun-HC is 5113.85; found 5114.16

Amino Acid Analysis

PFos: Asx (3) 3.29, Glx (10) 10.19, Ser (3) 2.60, Gly (2) 2.37, Thr (4) 4.35, Ala (5) 4.77, Ile (2) 1.37, Leu (8) 7.55, Phe (1) 0.77, Lys (4) 4.52.

PJun: Asx (2) 2.24, Glx (8) 8.50, Ser (4) 4.28, Gly (3) 3.32, Arg (3) 2.56, Thr (2) 1.90, Ala (7) 6.43, Val (3) 3.31, Met (1) 0.74, Ile (1) 0.96, Leu (5) 4.70, Lys (5) 4.90.

The results are consistent with the desired primary sequences.

Synthesis of 7-Hydroxycoumarin-3-carboxylic acid (1)

A mixture of 2.4g 2,4-dihydroxybenzaldehyde (20 mmol), 2.89g Meldrum's acid (20 mmol), piperidinium (0.4 mmol) and ethanol were stirred at room temperature for 20 minutes, and then refluxed for 2 hours. The reaction mixture was allowed to cool down to room temperature, followed by chilling in an ice bath for 30 minutes. The crystallized product was filtered, washed three times with ethanol, and dried in vacuum. Finally, ~3g yellow product (**1**) was collected.

Reference: Song, A; Wang, X; Lam, K. S.; *Tetra. Lett.*, **2003**, 44, 1755-8

Synthesis of Succinimido 3-Maleimidopropanoate (2).

β -Alanine (0.91g, 10mmol) was added to a solution of maleic anhydride (1.0g, 10mmol) in 12ml DMF, stirred for 2 hours at room temperature. When the entire solid was dissolved, an ice bath was used to decrease the temperature to 0 °C. N-Hydroxysuccinimide (1.44g, 12.5mmol) was added into the solution followed by DCC (4.12g, 20mmol). After 5-10 minutes, ice bath was removed and the reaction was kept at room temperature overnight, and white precipitate was obtained. The precipitate was washed with 60 ml water and 60 ml dichloromethane. The organic layer was washed with 40 ml water followed by 3 X 20ml 5% NaHCO₃ and finally, saturated NaCl solution. The organic layer was dried with Na₂SO₄ and the dichloromethane removed under reduced pressure. 1.6g solid (60% yield) was obtained.

Reference: Nicholas J. Ede, et al., *Bioconjugate Chem.*, **1994**, 5, 373-8

Synthesis of NHS ester of PAMAM dendrimer (Generation 0) (3).

0.5 g of 20 % w/w in methanol (0.07mmol) PAMAM dendrimer (Aldrich) was added into a round-bottom flask and methanol was removed under reduced pressure. 1ml DMSO was added into the flask to dissolve the dendrimer. 0.2g of (**2**) (0.75mmol) was dissolved in 1ml DMSO followed by 0.1g (0.78mmol) Diisopropylethylamine to make the activated ester. After 2 minutes, the activated ester was added into the flask which contained the dendrimer solution, and the flask was kept shaking at room temperature for 30 minutes. The red solution was acidified by acetic acid and purified by reverse-phase HPLC by a C18 column. 22mg of pure product was obtained. ESI analysis verified the molecular weight and proton NMR was used to verify the structure and purity (>95%) of the product.

Mass Spectroscopy (ESI) calculated for compound (2) is 1121.16; Found: 1121.3.

¹H NMR (DMSO): δ 2.3 (t, 8H, J=7.5Hz), 2.48 (m, 12H), 3.04 (m, 24H), 3.58 (t, 8H, J=7.5Hz), 6.99 (s, 8H), 8.00 (s, 4H), 8.12 (s, 4H).

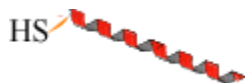
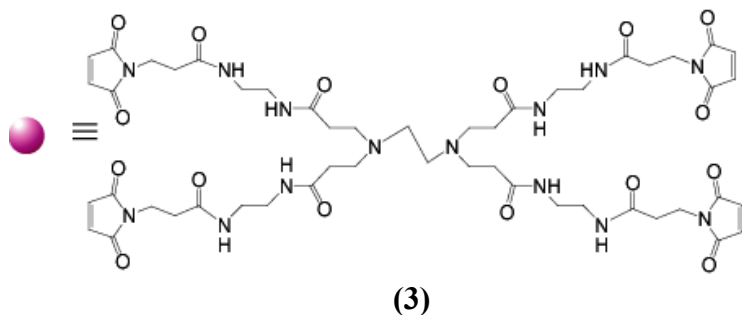
Synthesis of Dendrimer (D₀-Fos₄) (4)

D₀-Fos₄ was synthesized starting with 9.8 mg of PFos dissolved in 5mL buffer (0.1M Phosphate, pH = 7.5). 0.5 mg **3** (0.45 μmol) was added to the PFos solution. Argon was filled into the container to prevent disulfide bond formation. The reaction was kept overnight in a 37°C oven. The product (**4**) in the solution was purified by reverse-phase HPLC by a C18 column. Peptides were purified with a linear gradient of 20% to 80% acetonitrile containing 0.1% TFA at a flow rate of 8ml/min, for 60 minutes. ~2mg of (**4**) (yield ~ 24%) was obtained and identified by MALDI (see below).

Reinjection of purified D₀-Fos₄ onto a C18 analytical column verified that it were greater than 95% pure.

Mass Spectroscopy (MALDI) of the D₀-Fos₄:

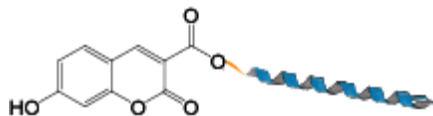
MALDI calculated for D₀-Fos₄ (M + H⁺) is 20298.80. Found: 20300.49



PFos: NH₂-CGSGSLTDTLQAETDQLEDEKXSALQTEIANLLKEKEKLEFILAA-CONH₂



PJun: NH₂-RIARLEEKVKTLKAQNSELASTANMLREQVALKQKVAASGSGCG-CONH₂



PJun-HC:

7-Hydroxycoumarin-GGRIARLEEKVKTLKAQNSELASTANMLREQVALKQKVAASGSGCG-CONH₂

Figure S1. Pink ball used in Fig.1 of manuscript represents the maleimide modified PAMAM generation 0 dendrimer. The peptide PFos and PJun and their sequence are also shown.

Circular Dichroism (CD) Spectroscopy

CD spectra were recorded on an Aviv 62A-DS spectropolarimeter. All measurements reported were carried out in pH 7.0 buffer (10 mM phosphate, 100mM NaCl, 1mM DTT), using a cuvette with a 1mm pathlength.

Peptide concentrations were determined from the following method reported for quantitative measurement of protein thiol groups, and verified by amino-acid analysis.

Reagents

DTNB (Ellman's reagent), 10mM (4mg/ml) in absolute methanol

Tris base (20mM)-EDTA (50mM) buffer, pH 8.0

Procedure

1. 130µl buffer + 15µl water + 5µl DTNB; absorbance B was measured at 412 nm;
2. 130µl buffer + 15µl water + 5µl peptide stock solution; absorbance C was measured at 412 nm;
3. 130µl buffer + 10µl water + 5µl DTNB + 5ml peptide stock solution; absorbance A was measured at 412 nm.
4. $[\text{peptide}] \mu\text{M} = (A - B - C) / 13600 \times (150/5) \times 10^6$

Reference: Hu, Miao-Lin, *Methods in Enzymology*, vol. 233, 381-5

Dendrimer-peptide hybrid concentrations were determined by amino-acid analysis.

All CD experiments were conducted at 25 °C and under Argon to minimize peptide exposure to atmosphere in order to prevent disulfide bond formation. Ellmans test was performed before and after each experiment to insure the absence the disulfide bonds.

Fluorescence Titration Utilizing the Gellman Assay

Fluorescence spectra were recorded on PTI fluorimeter (814 photomultiplier detection system and LPS-220B lamp power supply). All measurements reported were carried out at 25 °C and in a pH 7.0 buffer (10mM phosphate, 100mM NaCl, 1mM DTT). In each experiment, the concentration of PJun-HC is 1.67 µM. The concentration of D₀-Fos₄ is from 0 to 5µM (which is from 0 to 20 µM based on the PFos peptide). All the sample solutions were excited at 386 nm and the fluorescence emission was recorded from 400-600 nm. The maximal emission is at 447 nm. The following curves in Figure S2 individually represent the fluorescence emission of PJun-HC (1.67 µM, purple circle), D₀-Fos₄ (5 µM, green circle) and PFos (20 µM, red circle).

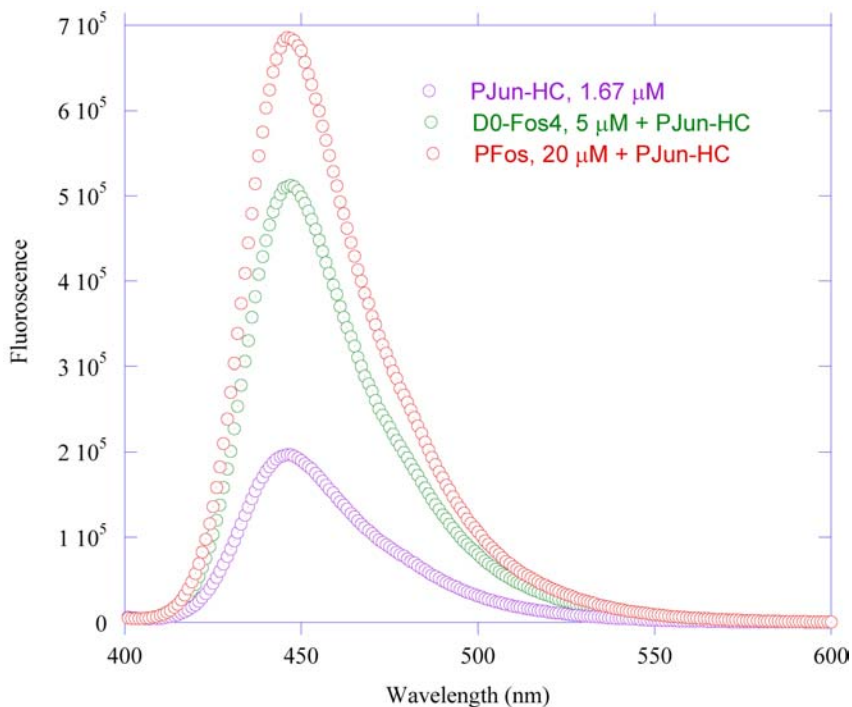


Figure S2. Fluorescence emission of PJun-HC (1.67 μM), D₀-Fos₄ (5 μM) and Pfos (20 μM). All samples were made in pH 7 buffer (10 mM phosphate, 100 mM NaCl, 1mM DTT), and excited at 386 nm.

Sedimentation Equilibrium Analysis

Materials and Methods

All sedimentation experiments were conducted on Beckman Optima XL-1 ultracentrifuge. All samples were made in pH 7.0 buffer with 10mM phosphate, 100mM NaCl and 1mM DTT (buffer A), and the same buffer A was used for overnight dialysis. Concentrations were determined by a combination of amino acid analysis and DTNB assays. Equilibrium at each speed was judged to be complete when three consecutive scans taken at two-hour intervals were indistinguishable from each other. Typical equilibrium experiments at each speed were carried out over 24 hours at a particular rotor speed. Data was analyzed using SEDEQ 4.1 (Allen Minton, Laboratory of Biochemical Pharmacology, NIH).

Result, Data Analysis and Discussion

1. D₀-Fos₄

40 μl of D₀-Fos₄ stock (75.74 μM) was mixed with 210 μl buffer and dialysis in 250 ml of the same buffer overnight. In the data analysis (rotor speed 15k rpm), the molecular weight was unconstrained, and 20.2 kD of molecular weight was obtained, which is consistent to the calculated molecular weight of 20.3 kD.

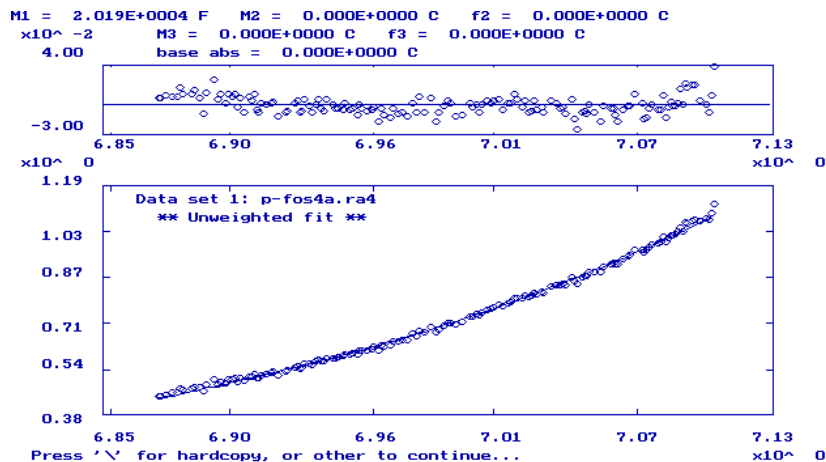


Figure S3. Sedimentation equilibrium result of D₀-Fos₄.

2. PJun and PJun-HC

58 μ M, 29 μ M and 19.33 μ M of PJun in pH 7 bufferA (after dialysis) were measured to calculate the K_d of PJun (rotor speed 25k). The unconstrained fits resulted in fits to 4.8 kD and 9.6 kD, following which in each experiment, the molecular weight were constrained to 4.8 kD (monomer) and 9.6 kD(dimer) of PJun. From the resulting ratios of monomer: dimer, the dissociation constant, K_d , was calculated as 45 μ M. Similar experiments with PJun-HC also showed only monomers and dimers, which gave a K_d of 13 μ M in agreement with results reported by Gellman and coworkers.

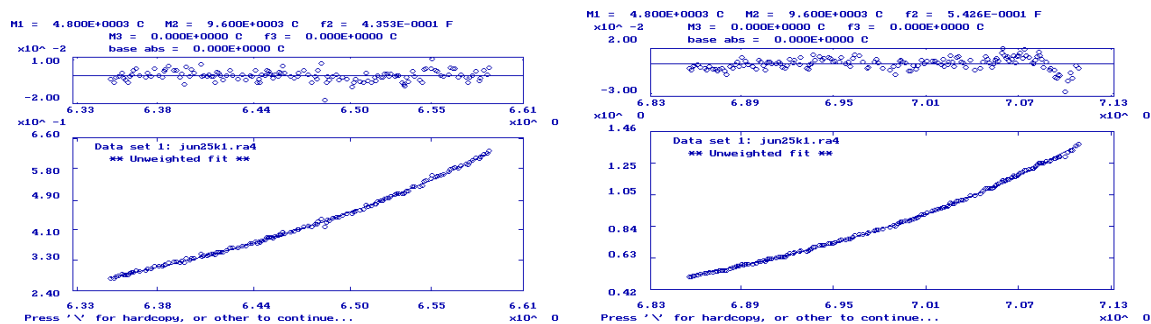


Figure S4. Sedimentation equilibrium result of PJun shown for concentrations of 29 and 58 μ M

3. D₀-Fos₄ + 4 equivalents of PJun

D₀Fos₄ + 4 equivalents of PJun: 34.3 μ l D₀-Fos₄ stock solution (75.7 μ M) was mixed with 14.63 μ l PJun stock solution (820 μ M), and diluted to 300 μ l with pH 7.0 bufferA. The sample was heated 10 minutes at 70~80 $^{\circ}$ C (over the T_m) and cooled to room temperature,

filtered and dialyzed in 250 ml of the same buffer overnight. The following table (S1) indicated all combinations of 3 components systems utilized to fit the equilibrium data (Figure S5.). We limited the fits to 3 components based on the principle of Ockam's Razor as the minimum number of variables that fit the data are judged to suitably explain the observed equilibrium and are in agreement with all other experimental data. The M.W. of 40 kD indicated the complex that 1 molecule of D₀-Fos₄ binds 4 molecules of PJun, and 30 kD indicated the complex that 1 molecule of D₀-Fos₄ binds 2 molecules of PJun. 20 kD is the M.W. of D₀-Fos₄. 4.8 kD is the M.W. of PJun monomer and 9.6 kD represents dimer.

Table S1

Curve Fits	M.W. (kD)	Fraction	Conclusion
1	40 30 4.8	0 0.764 0.236	No 40 kD and No PJun dimer makes this fit inconsistent.
2	40 30 9.6	0 0.709 0.293	No 40 kD and no PJun monomer dimer make this fit inconsistent.
3	40 9.6 4.8	0.479 0.525 -0.004	No PJun monomer observed make this fit inconsistent.
4	30 9.6 4.8	0.739 0.127 0.134	Most consistent result and in agreement with CD and fluorescence experiments
5	20 9.6 4.8	-0.06 -0.11	Meaningless as negative fractions obtained, and curve fits and residuals do not agree with the data

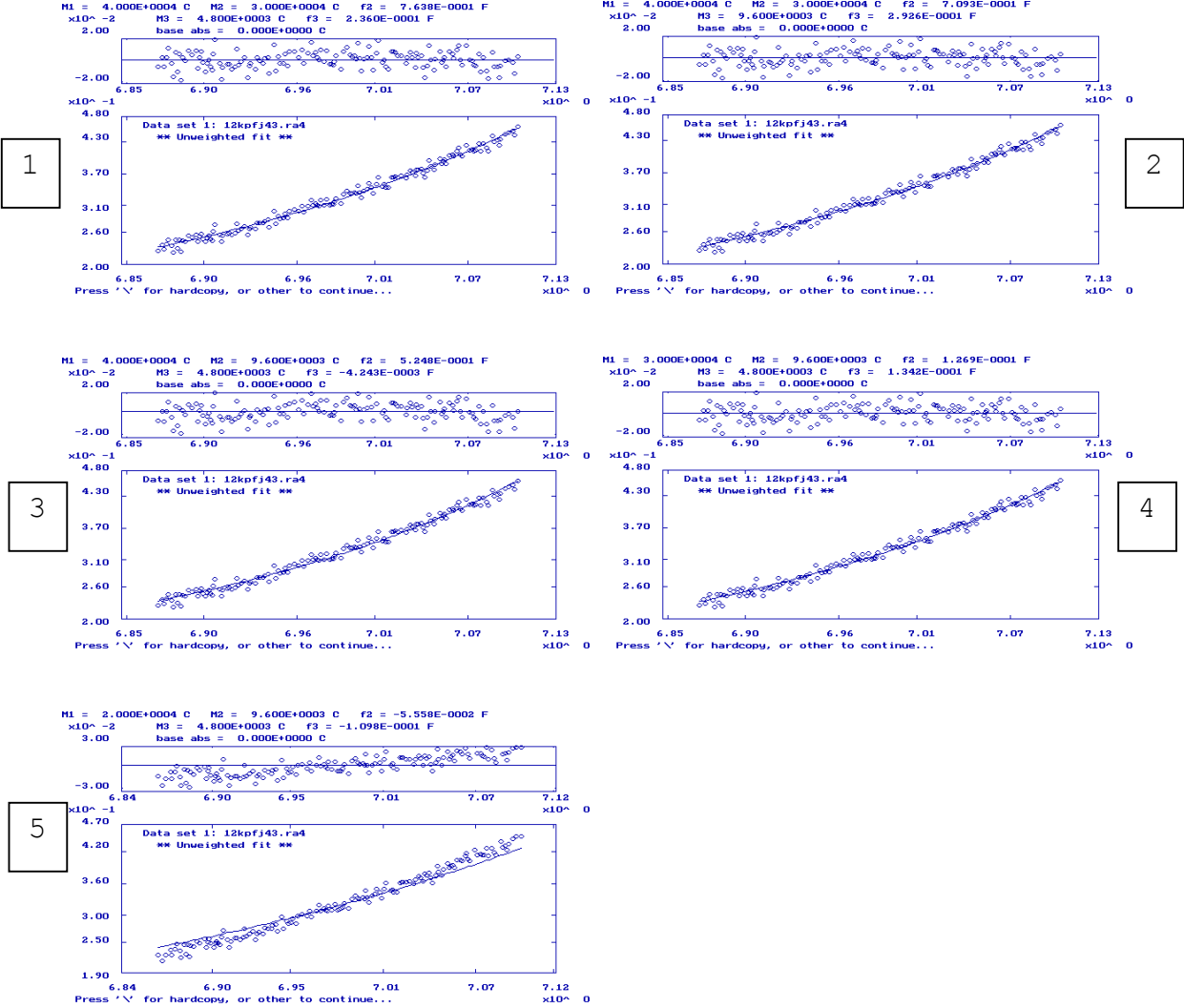


Figure S5. Sedimentation equilibrium data analysis of D₀-Fos₄ mixed with 4 fold of PFos. Fits are shown for 1-5 as described in Table S1.

4. D₀-Fos₄ + 8 equivalents of PJun

D₀Fos₄ + 8 equivalents of PJun: 42.2 μl D₀-Fos₄ stock solution (75.7 μM) was mixed with 34.5 μl PJun stock solution (743 μM), and diluted by 323 μl pH 7.0 buffer to make 400 μl solution. The sample was heated 10 minutes at 70~80 °C, cooled to room temperature, filtered and dialyzed in 250 ml of the same buffer overnight. The following table indicated all combinations of the 3 components system by which we tried to fit the data (see Figure S6).

Table S2

FITS	M.W. (kD)	Fraction	Conclusion
1	40 30 4.8	0.243 0.650 0.107	Fraction of complex is too small based upon known CD and fluorescence experiments. The fractions for PJun monomer/dimer ratio are not in agreement.
2	40 30 9.6	0.230 0.643 0.127	Fraction of complex is too small based upon known CD and fluorescence experiments. The fractions for PJun monomer/dimer ratio are not in agreement.
3	40 9.6 4.8	0.684 0.201 0.115	Best Fit and fractions of PJun are in agreement to PJun monomer/dimer ratio determined for PJun alone and also in agreement with CD and fluorescence data.
4	30 9.6 4.8	2.464 0.037 -1.501	Fractions are nonsensical
5	20 9.6 4.8	1.113 -0.087 -0.026	Fractions are nonsensical and curve fits and residuals do not fit the data

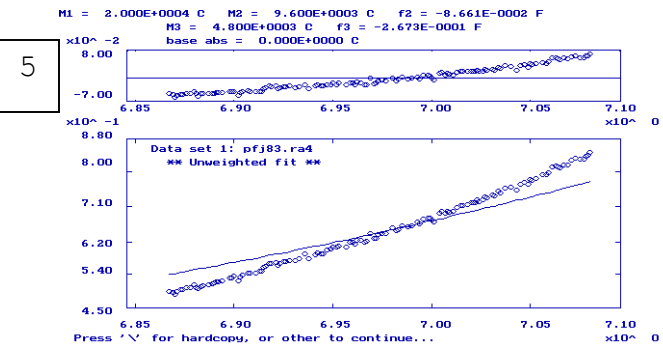
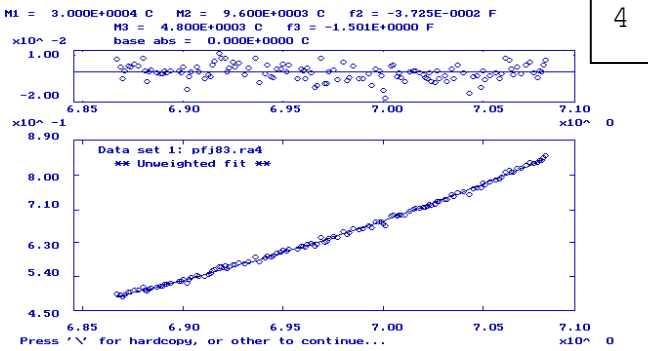
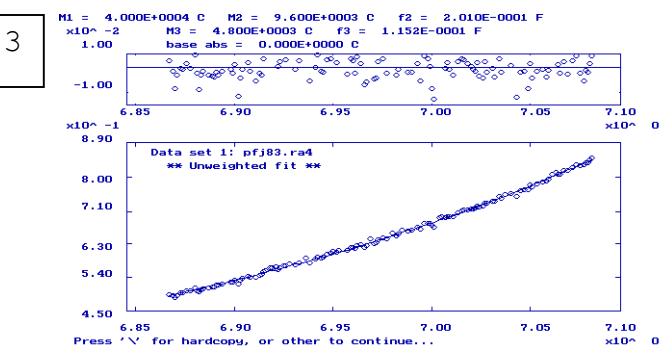
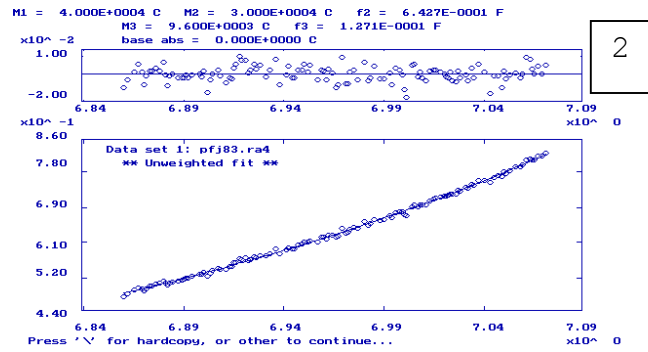
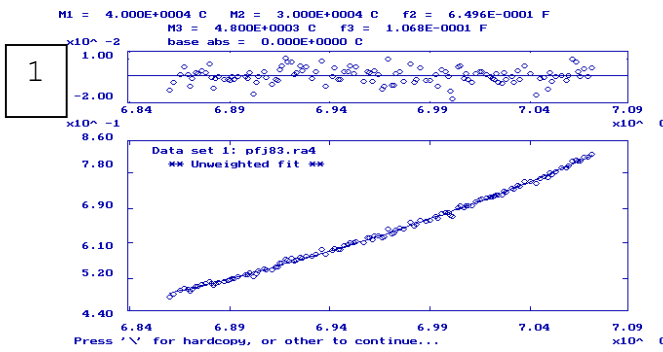


Figure S6. Sedimentation equilibrium data analysis of D₀-Fos₄ mixed with 8 equivalents of PFos. Fits are shown for 1-5 as described in Table S2.

Unconstrained Fit for P₀Fos₄/4Jun

Finally, we unconstrained the molecular weight of the assembled complex, and we also know there is excess PJun in solution, so PJun (MW 4.8 kD) and homodimer of 2.PJun (MW 9.6 kD) must show up in the solution as seen in CD and sedimentation equilibrium experiments. Thus we constrained these two molecular weight parameters. The resulting fit with this constraint showed a very good fit with evenly distributed residuals. The experimental MW of the assembled complex was found to be 41.56 kD, is in close agreement to the theoretical MW (39.5 kD) of the complex P₀Fos₄/4Jun.

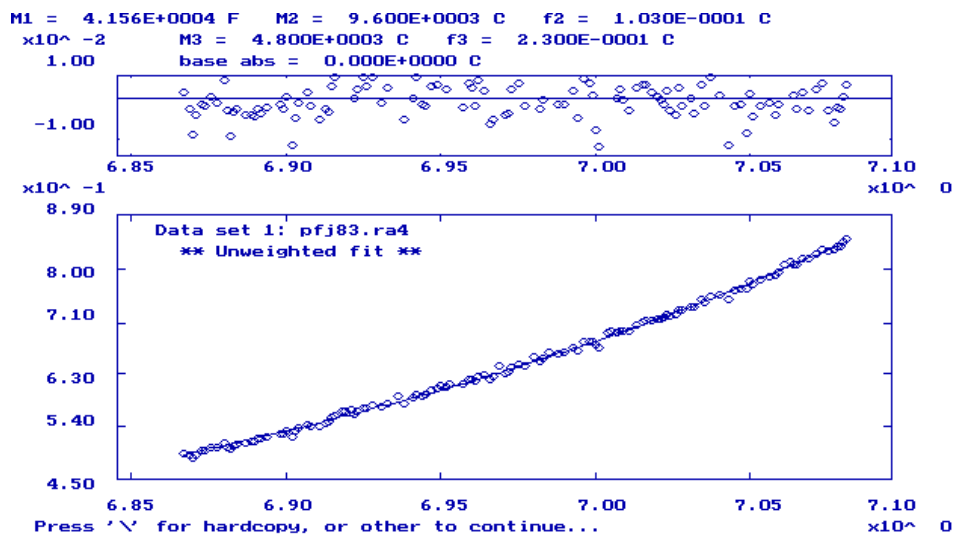


Figure S7. Unconstrained Fit for the sedimentation equilibrium data analysis of D₀-Fos₄ mixed with 8 equivalents of PFos