## Noncovalent Multivalent Assembly of Jun Peptides on a Leucine Zipper Dendrimer Displaying Fos Peptides

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Received July 27, 2004



ABSTRACT

The synthesis and characterization of a new leucine-zipper dendrimer (LZD) is reported that displays four copies of the peptide corresponding to the coiled-coiled dimerization domain of Fos. Circular dichroism spectroscopy, fluorescence titration, and sedimentation equilibrium experiments demonstrate that Fos-LZD can noncovalently assemble four copies of the peptide corresponding to the coiled-coil domain of Jun. This work provides the basis for the future construction of noncovalently assembled multivalent protein assembles displaying any protein of interest.

There is much interest in utilizing noncovalent interactions to control the biomimetic assembly of unimolecular, multimeric, and polymeric structures for biological and nanotechnological applications.<sup>1</sup> Many elegant unimolecular, conformationally unique structures have been recently designed including  $\beta$ -peptides<sup>2</sup> and template-assembled synthetic proteins (TASPs) and other novel foldamers.<sup>3</sup> At the same time more complex polymeric self-assembling structures have also been designed, which include cyclic-peptide nanotubes,<sup>4</sup> peptide-hydrogels,<sup>5</sup> and even PNA-microgels.<sup>6</sup> Dendrimer construction has also witnessed the use of self-

*Chem. Rev.* **2001**, *101*, 3893–4011. (b) Mutter, M.; Vuilleumier, S. Angew. Chem., Int. Ed. Engl. **1989**, *28*, 535–554.

assembly utilizing either hydrogen bonding<sup>7</sup> or metal coordination.<sup>8</sup> We have recently combined aspects of peptide selfassembly and dendrimeric architecture in the synthesis of leucine zippers appended to a core dendrimer, providing the necessary framework for constructing multivalent leucine zipper dendrimers (LZDs).<sup>9</sup> These LZDs can noncovalently assemble cognate helical peptides at their periphery. The ability to design biomimetic modular multivalent systems is of much interest in targeting cellular receptors,<sup>10</sup> DNA,<sup>11</sup> and in assembling novel architectures.<sup>1,3</sup>

We have focused on leucine zippers or, more generically, helical coiled-coils<sup>12</sup> as our self-assembly unit as they are very amenable to design<sup>13</sup> and many orthogonal pairs exist

(8) Kawa, M.; Frechet, J. M. J. Chem. Mater. 1998, 10, 286-296

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<sup>(1)</sup> Lindsey, J. S. New J. Chem. 1991, 15, 153-180.

<sup>(2)</sup> Appella, D. H.; Christianson, L. A.; Klein, D. A.; Powell, D. R.;
Huang, X.; Barchi, J. J., Jr.; Gellman, S. H. *Nature* **1997**, *387*, 381–384.
(3) (a) Hill, D. J.; Mio, M. J.; Prince, R. B.; Hughes, T. S.; Moore, J. S.

<sup>(4)</sup> Ghadiri, M. R.; Granja, J. R.; Milligan, R. A.; McRee, D. E.; Khazanovich, N. *Nature* **1993**, *366*, 324–327.

<sup>(5) (</sup>a) Petka, W. A.; Harden, J. L.; McGrath, K. P.; Wirtz, D.; Tirrell, D. A. *Science* **1998**, *281*, 389–392. (b) Schneider, J. P.; Pochan, D. J.; Ozbas, B.; Rajagopal, K.; Pakstis, L.; Kretsinger, J. J. Am. Chem. Soc. **2002**, *124*, 15030–15037.

<sup>(6)</sup> Cao, R.; Gu, Z. Y.; Hsu, L.; Patterson, G. D.; Armitage, B. A. J. Am. Chem. Soc. 2003, 125, 10250–10256.

<sup>(7)</sup> Zimmerman, S. C.; Zeng, F. W.; Reichert, D. E. C.; Kolotuchin, S. V. *Science* **1996**, *271*, 1095–1098.

<sup>(9)</sup> Zhou, M.; Bentley, D.; Ghosh, I. J. Am. Chem. Soc. 2004, 126, 734–735.

<sup>(10) (</sup>a) Mammen, M.; Choi, S. K.; Whitesides, G. M. Angew. Chem., Int. Ed. 1998, 37, 2755–2794. (b) Kiessling, L. L.; Gestwicki, J. E.; Strong, L. E. Curr. Opin. Chem. Biol. 2000, 4, 696–703. (c) Gestwicki, J. E.; Cairo, C. W.; Strong, L. E.; Oetjen, K. A.; Kiessling, L. L. J. Am. Chem. Soc. 2002, 124, 14922–14933.

<sup>(11)</sup> Kim, J. S.; Pabo, C. O. Proc. Natl. Acad. Sci. U.S.A. 1998, 95, 2812–2817.

naturally<sup>14</sup> or can be selected.<sup>15</sup> This potentially allows for the construction of numerous orthogonal self-assembling systems, which is not possible with currently utilized streptavidin—biotin<sup>6,16</sup> or Ni-chelation<sup>17</sup> based multivalent assembly systems. Helical coiled-coils organize their amino acids in a repeating heptad pattern, which when numbered a—g places a specific hydrophobic residue, such as leucine, at the a and d positions to create the knob-hole like hydrophobic interface.

Our previous design strategy utilized electrostatically driven complexation of a coiled-coil tetramer upon the generation-0 PAMAM dendrimeric core at either low or high pH regimes. To expand the utility of our approach toward the facile assembly of discrete multivalent supramolecules at neutral pH, herein we report the synthesis and characterization of a new LZD that is functional at neutral pH. This new LZD displays the coiled-coil domain of the human transcription factor, Fos, and assembles four copies of a peptide corresponding to the coiled-coil domain of its cellular binding partner Jun (Figure 1). Such a system can have great



**Figure 1.** PFos (red) is shown appended to the generation-0 PAMAM dendrimer (purple ball) to result in the LZD  $D_0$ -Fos<sub>4</sub>. The sequences of PFos (red), PJun (blue), and 7-hydroxycoumarinappended PJun (PJun-HC) are also shown.

utility in the noncovalent multivalent display of any protein fused to a Jun-coiled-coil. This system is especially attractive as the Fos/Jun coiled-coil pair has been shown to be

(16) Green, N. M. Method Enzymol. **1990**, 184, 51–67.

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orthogonal to a multitude of other natural coiled-coil pairs<sup>14</sup> and provides a rational starting point for the future assembly of multiple mutually exclusive LZD/cargo pairs. Multiple designed leucine zippers, as utilized in our previous design, have yet to be systematically tested for orthogonality in competitive cellular environments.

In our design, peptides corresponding to the coiled-coil domains of the well-characterized bZIP protein Fos and Jun<sup>18,19</sup> were chosen as starting points as our self-assembling units. We anticipated that the covalent attachment of four coiled-coils to a dendrimer would result in the undesirable intramolecular stabilization of coiled-coils, which is useful in TASP assemblies.<sup>3</sup> We reasoned that the less stable Fos coiled-coil would be suitable for decorating the core dendrimer on the basis of literature precedence that the Fos/Fos homodimer is less stable than the Jun/Jun homodimer, both of which are an order of magnitude less stable than the Fos/Jun heterodimer.<sup>18,19</sup>

The peptides corresponding to the coiled-coil domains of Fos and Jun, PFos and PJun were synthesized by standard solid-phase Fmoc chemistry on a Rink amide resin. Peptides were cleaved and purified by HPLC and subsequently characterized by amino acid analysis and MALDI mass spectrometry (see Supporting Information). The maleimidofunctionalized generation-0 PAMAM dendrimer was reacted chemoselectively with a unique Cys at the N-terminus of the Fos peptide to generate  $D_0$ -Fos<sub>4</sub> in 20-25% yield (Supporting Information). We also synthesized the Jun peptide (PJun), with an added 7-hydroxycoumarin (PJun-HC) followed by a Gly-Gly linker at the N-terminus. PJun-HC was synthesized to monitor the noncovalent assembly of PFos/PJun-HC and D<sub>0</sub>-Fos<sub>4</sub>/4PJun-HC complexes utilizing the Gellman assay,18 which monitors the relief of excimermediated quenching of the PJun-HC homodimer. The respective sequence of the peptides and LZD-dendrimer used in this study are shown in Figure 1.

Our initial studies focused on characterization of D<sub>0</sub>-Fos<sub>4</sub> in comparison to PFos. We predicted that D<sub>0</sub>-Fos<sub>4</sub> would fold into two coiled-coils (Figure 1) as a disulfide linked Fos-peptide homodimer has been previously been shown to be completely folded.<sup>19</sup> We utilized circular dichroism (CD) to monitor the secondary structure of the dendrimer D<sub>0</sub>-Fos<sub>4</sub> in comparison to PFos at pH 7 with 10 mM phosphate, 100 mM NaCl, 1 mM DTT (buffer A). The CD spectra of PFos and the LZD, D<sub>0</sub>-Fos<sub>4</sub>, were significantly different (Figure 2a) and indicated that PFos is primarily unfolded at 25 °C, whereas the D<sub>0</sub>-Fos<sub>4</sub> is 85% folded under the same conditions. The greater helicity of D<sub>0</sub>-Fos<sub>4</sub> is likely due to the anticipated covalent stabilization as reported for the disulfidetethered Fos peptides and also observed in coiled-coil assembly in the seryl tRNA synthetase.<sup>20</sup> To interrogate the stability of D<sub>0</sub>-Fos<sub>4</sub> in comparison to PFos, we monitored the temperature-dependent change in secondary structure

<sup>(12) (</sup>a) Crick, F. H. C. Acta Crystallogr. **1953**, 6, 689–697. (b) Lupas, A. Trends Biochem. Sci. **1996**, 21, 375–382.

<sup>(13) (</sup>a) Oshea, E. K.; Lumb, K. J.; Kim, P. S. *Curr. Biol.* 1993, *3*, 658–667. (b) Monera, O. D.; Kay, C. M.; Hodges, R. S. *Biochemistry* 1994, *33*, 3862–3871. (c) Bilgicer, B.; Fichera, A.; Kumar, K. *J. Am. Chem. Soc.* 2001, *123*, 4393–4399. (d) Schnarr, N. A.; Kennan, A. J. *J. Am. Chem. Soc.* 2001, *123*, 11081–11082.

<sup>(14)</sup> Newman, J. R. S.; Keating, A. E. Science 2003, 300, 2097-2101.
(15) (a) Arndt, K. M.; Pelletier, J. N.; Muller, K. M.; Alber, T.; Michnick, S. W.; Pluckthun, A. J. Mol. Biol. 2000, 295, 627-639. (b) Kim, B. M.; Oakley, M. G. J. Am. Chem. Soc. 2002, 124, 8237-8244. (c) Ghosh, I.; Hamilton, A. D.; Regan, L. J. Am. Chem. Soc. 2000, 122, 5658-5659.

<sup>(17)</sup> Griffith, B. R.; Allen, B. L.; Rapraeger, A. C.; Kiessling, L. L. J. Am. Chem. Soc. **2004**, *126*, 1608–1609.

<sup>(18)</sup> Daugherty, D. L.; Gellman, S. H. J. Am. Chem. Soc. **1999**, 121, 4325–4333.

<sup>(19) (</sup>a) O'Shea, E. K.; Rutkowski, R.; Stafford, W. F. D.; Kim, P. S. *Science* **1989**, *245*, 646–648. (b) Kohler, J. J.; Schepartz, A. *Biochemistry* **2001**, *40*, 130–142.

<sup>(20)</sup> Oakley, M. G.; Kim, P. S. Biochemistry 1997, 36, 2544-2549.



**Figure 2.** CD secondary structural analysis of PFos and  $D_0$ -Fos<sub>4</sub>. (a) CD spectra of 20  $\mu$ M PFos (red) and 5  $\mu$ M dendrimer  $D_0$ -Fos<sub>4</sub> (green) at 25 °C in pH 7.0 buffer containing 10 mM phosphate, 100 mM NaCl, and 1mM DTT (buffer A). (b) Thermal unfolding of 20  $\mu$ M PFos and 5  $\mu$ M  $D_0$ -Fos<sub>4</sub> in buffer A followed by the change in CD signal at 222 nm.

(Figure 2b) by following the change at  $[\theta]_{222}$  by CD spectroscopy.

In the CD melting curve, the apparent  $T_{\rm m}$  (where 50% of the structures are folded) of  $D_0$ -Fos<sub>4</sub> at 5  $\mu$ M (20  $\mu$ M based on PFos concentration) is  $\sim 40$  °C higher than that of PFos at 20  $\mu$ M. The mean residue ellipticity at  $[\theta]_{222}$  of D<sub>0</sub>-Fos<sub>4</sub> at 4 and 96 °C was taken to represent the theoretical maximum folded and unfolded baselines for D<sub>0</sub>-Fos<sub>4</sub> and PFos at these concentrations. Given the relative stability of D<sub>0</sub>-Fos<sub>4</sub> we wanted to ensure that it did not form higher order oligomers that would complicate analysis for PJun complexation studies. Toward this end we measured the molecular weight and oligomerization states of the PJun, PJun-HC, and D<sub>0</sub>-Fos<sub>4</sub> utilizing sedimentation equilibrium (Supporting Information). Measurements were made on an analytical ultracentrifuge at 20 °C in PBS at three different concentrations. We found that PJun and PJun-HC existed in equilibrium between monomer and dimer, whereas D0-Fos4 existed only as a monomer. The dissociation constant of PJun was found to be 45  $\mu$ M, which was 3.5-fold weaker than that for PJun-HC, which was found to be 13  $\mu$ M.

Gellman and co-workers have previously reported a similar increase in stability of a Jun-based peptide homodimer upon the incorporation of 7-hydroxycoumarin.<sup>18</sup> Having established that PJun was only partially helical between 0 and 50  $\mu$ M and that D<sub>0</sub>-Fos<sub>4</sub> was completely helical and monomeric, we predicted that it would be possible to monitor the assembly of four copies of peptide Jun upon the LZD D<sub>0</sub>-Fos<sub>4</sub> utilizing CD spectroscopy. In our CD experiment, we assumed that the overall helicity of PJun would increase as it was complexed by  $D_0$ -Fos<sub>4</sub>. To test this, we made a series of samples in pH 7.0 buffer (10 mM phosphate, 100 mM NaCl, 1 mM DTT), such that the concentration of  $D_0$ -Fos<sub>4</sub> was constant at  $2 \mu M$  (8  $\mu M$  based on PFos concentration), while the concentration of PJun was varied from 0 to 40  $\mu$ M in the presence and absence of D<sub>0</sub>-Fos<sub>4</sub>. The results for  $2 \mu M D_0$ -Fos<sub>4</sub>, 16  $\mu M$  PJun, and  $2 \mu M$  of  $D_0$ -Fos<sub>4</sub> + 16  $\mu M$ PJun is shown in Figure 3a. It is clear that  $2 \mu M D_0$ -Fos<sub>4</sub> is



**Figure 3.** CD analysis of D<sub>0</sub>-Fos<sub>4</sub> and PJun complex formation. (a) The uncorrected CD spectra of 2  $\mu$ M D<sub>0</sub>-Fos<sub>4</sub> (green circle), 16  $\mu$ M PJun (purple circle), and 2  $\mu$ M D<sub>0</sub>-Fos<sub>4</sub> + 16  $\mu$ M PJun (blue circle). The curve (black cross) represents the calculated weighted average spectra for 2  $\mu$ M D<sub>0</sub>-Fos<sub>4</sub> + 16  $\mu$ M PJun. (b) Change in PJun helicity upon complexation with Do-Fos<sub>4</sub> at [ $\theta$ ]<sub>222</sub> in the presence of 2  $\mu$ M D<sub>0</sub>-Fos<sub>4</sub>. Change in  $\theta$ <sub>222</sub> of PJun is calculated from ( $\theta$ <sub>222</sub>)<sub>[D0-Fos<sub>4</sub>+PJun]</sub> – ( $\theta$ <sub>222</sub>)<sub>[PJun]</sub> – ( $\theta$ <sub>222</sub>)<sub>[D0-Fos<sub>4</sub>]</sub> All samples were in buffer A.

fully folded, and 16  $\mu$ M PJun is primarily unfolded. If there were no interaction between D<sub>0</sub>-Fos<sub>4</sub> and PJun, then the experimental spectra of  $2 \mu M D_0$ -Fos<sub>4</sub> with 16  $\mu M$  Jun would be equivalent to the sum of the spectra for  $D_0$ -Fos<sub>4</sub> (2 $\mu$ M) and PJun (16  $\mu$ M). However, the CD spectra clearly show that 16  $\mu$ M PJun + 2  $\mu$ M of D<sub>0</sub>-Fos<sub>4</sub> is considerably more helical than that for the calculated addition spectra. This change in helicity can be directly attributed to change in PJun secondary structure upon complexation with D<sub>0</sub>-Fos<sub>4</sub>. The result of the complete CD titration experiments is shown in Figure 3b, where the relative change in PJun helicity at 222 nm obtained from the subtraction spectra of  $(\theta_{222})_{[D_0 - Fos_4 + PJun]}$  $-(\theta_{222})_{[PJun]} - (\theta_{222})_{[D_0 - Fos_4]}$  is shown as a function of increasing PJun concentration. The titration curve plateaus at 16  $\mu$ M PJun, suggesting that an 8-fold excess (16  $\mu$ M) of PJun is required for the complete complexation of 2  $\mu$ M D<sub>0</sub>-Fos<sub>4</sub>, where all the PFos tethered to the LZD is completely occupied by PJun. This analysis, although confirming our design strategy, is complicated by the concentration-dependent monomer-dimer equilibrium of PJun.

We further evaluated the complexation of  $D_0$ -Fos<sub>4</sub> with PJun utilizing the Gellman assay,<sup>18</sup> where titration of PJun-HC with either  $D_0$ -Fos<sub>4</sub> or PFos is expected to lead to an increase in PJun-HC fluorescence due to an increase in the average distance between the 7-hydroxycoumarins attached to dimeric PJun-HC. The change in PJun-HC fluorescence was monitored by excitation at 386 nm and emission at 447 nm. The concentration of PJun-HC was held constant at 1.67  $\mu$ M, while the concentration of  $D_0$ -Fos<sub>4</sub> (0 to 5  $\mu$ M) or PFos (0 to 20  $\mu$ M) was varied. The results are shown in Figure 4a, where the change in PJun-HC fluorescence is plotted with respect to the molar ratio of [PFos]/[PJun-HC], ([D\_0-Fos<sub>4</sub>])/ [PJun-HC]. The results from the PJun-HC titration clearly show that both D<sub>0</sub>-Fos<sub>4</sub> and PFos can complex PJun-HC, resulting in relief of excimer quenching as the 7-hydroxy-



**Figure 4.** Fluorescence titration of PJun-HC. In (a) the titration of PJun-HC by PFos ( $\blacklozenge$ ) and D<sub>0</sub>-Fos<sub>4</sub> ( $\bigcirc$ ) is shown. In (b) the same data are shown, but D<sub>0</sub>-Fos<sub>4</sub> concentration is expressed as [D<sub>0</sub>-Fos<sub>4</sub>]/4 ( $\Box$ ) to compare more directly with PFos ( $\blacklozenge$ ) on a per leucine zipper basis. PJun-HC was constant at 1.67  $\mu$ M; excitation was at 386 nm, and emission recorded at 447 nm.

coumarins are no longer held in close proximity. Moreover, in both cases recovery of fluorescence plateaus when the concentration of PFos is over 6-fold that of PJun-HC. However, whereas PFos was observed to recover 50% of PJun-HC fluorescence at 0.7 molar equiv, D<sub>0</sub>-Fos<sub>4</sub> could only recover 50% of PJun-HC fluorescence at 1.9 molar equiv based on a per leucine zipper basis (Figure 4b). This 2.7fold difference in the ability of D<sub>0</sub>-Fos<sub>4</sub> versus PFos to successfully complex PJun-HC can be attributed to the energetic penalty for first having to unfold the covalently stabilized Fos peptides dimers on D<sub>0</sub>-Fos<sub>4</sub> prior to complexation with PJun-HC. Alternatively, it is possible that the assembly of four PJun peptides on the D<sub>0</sub>-Fos<sub>4</sub> is sterically demanding. It is also interesting to note that the absolute recovered fluorescence of PJun-HC was 25% less when titrated with D<sub>0</sub>-Fos<sub>4</sub> relative to PFos, which is very likely due to the four 7-hydroxycoumarins still being held in close proximity when decorated upon the D<sub>0</sub>-Fos<sub>4</sub> LZD scaffold (Supporting Information).

Sedimentation equilibrium was utilized to directly interrogate the  $D_0$ -Fos<sub>4</sub>/PJun complex formation. On the basis of our CD and fluorescence titration experiments we estimated that an 8-fold excess of PJun was required to complex all four sites on  $D_0$ -Fos<sub>4</sub>. Thus, in the sedimentation equilibrium experiments two different molar ratios were utilized where the  $D_0$ -Fos<sub>4</sub>/PJun was held at relative ratios of either 1:4 or 1:8. Analysis of the sedimentation equilibrium

data indicated that when the molar ratio of D<sub>0</sub>-Fos<sub>4</sub>/PJun was 1:4, the data was best fit by three species corresponding to D<sub>0</sub>-Fos<sub>4</sub>/2PJun, PJun-monomer and PJun dimer. However, when the molar ratio of  $D_0$ -Fos<sub>4</sub>/PJun was 1:8, the data was best fit to three species corresponding to D<sub>0</sub>-Fos<sub>4</sub>/4PJun, PJun-monomer, and PJun dimer (Supporting Information). Thus, these sedimentation equilibrium results along with the fluorescence and CD experiments indicate that we have successfully assembled four copies of PJun on the D<sub>0</sub>-Fos<sub>4</sub> scaffold to afford a noncovalent supramolecular complex of 40,000 Da in molecular weight. The complexation of PJun with D<sub>0</sub>-Fos<sub>4</sub> could follow a two-step process, where first two of the PFos peptides on D<sub>0</sub>-Fos<sub>4</sub> unfold and bind 2 equiv of PJun, following which the next two Fos peptides unfold and bind another 2 equiv of PJun. Alternatively, the PJun peptides could bind the Fos peptides appended to the dendrimer core one at a time, which does not agree with our sedimentation equilibrium data and would also be energetically unfavorable.

This work demonstrates the noncovalently assembly of four coiled-coil peptides upon a leucine zipper dendrimerdisplaying scaffold characterized by CD, fluorescence, and sedimentation equilibrium experiments. These studies also show that the significant covalent stabilization of leucine zippers by the dendrimeric scaffold has to be overcome for the successful noncovalent assembly of cognate peptides. Future work in this area will entail the expression of proteins directly attached to leucine zippers, such as PJun, such that they can be complexed upon LZDs, such as D<sub>0</sub>-Fos<sub>4</sub>, to instantly afford functional multivalent protein complexes. The orthogonality of different leucine-zipper pairs can potentially allow for the coexistence of many such assemblies that can be utilized for DNA binding,11 targeting receptors on cellsurfaces,<sup>10</sup> or in mimicking multivalent enzymatic complexes.21

Acknowledgment. We thank Tom Baldwin and Mike Cusanovich for the use of their instruments. This work was supported by the donors of the Petroleum Research Fund for a PRF Type G grant and by the Research Corporation for a Research Innovation Award.

**Supporting Information Available:** Synthesis, characterization, fluorescence and sedimentation equilibrium data for  $D_0$ -Fos<sub>4</sub>, PFos, PJun, and PJun-HC. This material is available free of charge via the Internet at http://pubs.acs.org.

## OL0485262

<sup>(21)</sup> Kumar, P.; Li, Q.; Cane, D. E.; Khosla, C. J. Am. Chem. Soc. 2003, 125, 4097–4102.