Antiparallel Leucine Zipper-Directed Protein Reassembly: Application to the Green Fluorescent Protein

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The dissection and subsequent reassembly of a protein from peptidic fragments provides an avenue for controlling its tertiary structure and hence its function. Here, we describe a general method for the reassembly of protein fragments mediated by the noncovalent association of antiparallel leucine zippers.[1] Although a majority of leucine zippers associate in a parallel fashion, recent examples of both naturally occurring and designed antiparallel leucine zippers have appeared in the literature.[1,2] We report here a strategy for the noncovalent reconnection of the N- and C-termini of a dissected surface loop of a protein by means of antiparallel leucine zippers (Figure 1).[3] We have successfully applied this oligomerization strategy, both in vitro and in vivo, to the 238 residue green fluorescent protein (GFP) from *Aequorea victoria*.[4] GFP provides an easily testable system for correct reassembly by virtue of its autocatalytically generated fluorescence, which is intimately linked to its properly folded structure.[5] Moreover, the current interest in utilizing GFP as a biosensor provides further motivation for generating new tools for biotechnological applications based on the strategy we describe.[5]

The unassisted reconstitution of proteins from peptide fragments has been demonstrated for several proteins; including ribonuclease,[6a] chymotrypsin inhibitor-2,[6b] tRNA synthetases,[6c] and inteins.[6d] Protein reassembly has thus become an important avenue for understanding enzyme catalysis,[6a] protein folding,[6b] and protein evolution.[6c] Recently, assisted protein reassembly or "fragment complementation" has been applied to the in vivo detection of protein−protein interactions in such systems as dihydrofolate reductase (DHFR),[7a−c] ubiquitin,[7d,e] and β-galactosidase.[7f] These reassembly processes are contingent upon the proper choice of a dissection site within a protein and can be aided by techniques such as limited proteolysis, circular permutation[8] and loop insertions.[9] In particular, recent circular permutation[8c] and protein insertion[8b,9] strategies have provided convincing evidence that GFP can fold, fluoresce, and serve as a biosensor despite the rearrangement of the natural coding sequence.

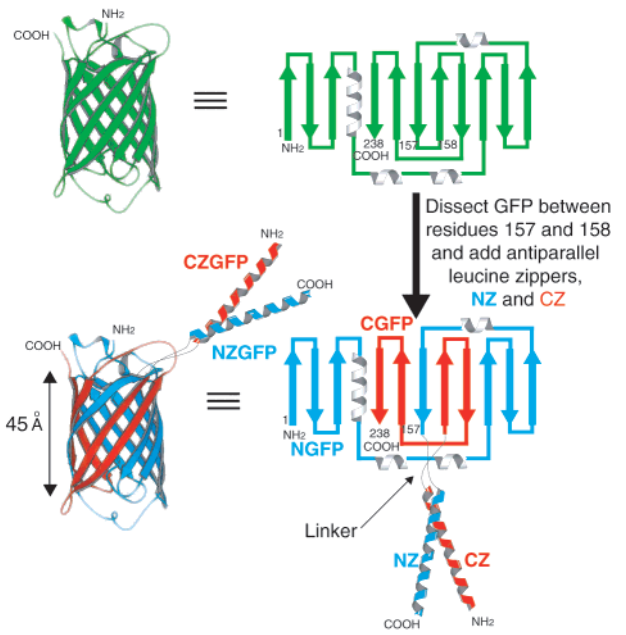

Figure 1. Strategy for antiparallel leucine zipper-directed protein reassembly of GFP.[3] Both the ribbon and topographical structures are depicted: GFP is shown in green, NZGFP is shown in blue and CZGFP is shown in red. The sequences of the designed leucine zippers, NZ and CZ, are ALKKELQANKKELAQLKWELQALKKELAQ and EQLEKKLQALEKKLAQLEWKNQALEKKLAQ, respectively.

In our study we have used a variant of the naturally occurring GFP, which has a single excitation maximum at 475 nm.[10] Our design strategy called for the dissection of GFP at a surface loop between residues 157 and 158, a position that has previously been shown to accommodate a 20-residue amino acid insertion.[9a] Our dissection resulted in N- and C-terminal fragments, designated NGFP and CGFP, containing 157 and 81 residues, respectively (Figure 1). The NGFP fragment contains the three residues, Ser65, Tyr66, and Gly67, that ultimately form the GFP fluorophore.[4] Designs for helices, designated NZ and CZ, to form antiparallel leucine zippers for reassembly purposes were based upon sequences reported by Hodges,[11a] Kim,[11b] and Alber.[11c] The leucine zippers contained a Leu-rich hydrophobic core, acidic (Glu) and basic (Lys) residues to direct antiparallel heterodimer formation, and also incorporated a buried asparagine residue which disfavors homodimerization by up to 2.3 kcal/mol (Figure 1).[2a] The designed helix, NZ was appended to the C-terminal of NGFP, via a 6-residue linker, to generate the fragment designated

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(10) The GFP variant (sg100) in this work was a gift from G. J. Palm and contains F64L, S65C, Q80R, Y151L, I167T, and K238N mutations from wild-type GFP, which leads to a single fluorescence excitation and emission maximum at 475 and 505 nm respectively, similar to GFP-sg25 as described in: Palm, G. J.; Zdanov, A.; Gaitanaris, G. A.; Stauber, R.; Pavlakis, G. N.; Wlodawer, A. *Nat. Struct. Biol.* **199**7, *4*, 361−365.

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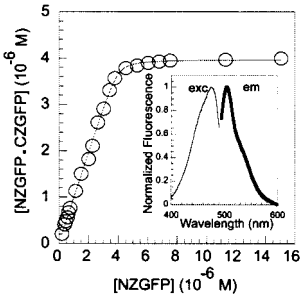

Figure 2. Fluorescence binding isotherm for the interaction of NZGFP with CZGFP monitored at 505 nm. Inset shows the normalized fluorescence excitation and emission of the reconstituted NZGFP·CZGFP complex.

NZGFP. Similarly, CZ was appended to the N-terminal residue of CGFP, via a 4-residue linker, to generate the complementary fragment, CZGFP. It was envisioned that if NZGFP and CZGFP were competent to heterodimerize via the designed helices, either in vitro or in vivo, the reconstituted GFP protein would display its characteristic fluorescence,[10] indicating the correct reassembly of the tertiary fold from the peptide fragments. The genes encoding the designed protein sequences NZGFP, CZGFP, NGFP, and CGFP were cloned and the resulting proteins overexpressed and purified using standard methods.

To investigate the viability of our protein reassembly strategy, we followed a literature protocol devised for the refolding of denatured GFP.[5b] Thus, equimolar amounts (4 μM) of the fragments, NZGFP and CZGFP, were denatured in 6 M GdmCl and dialyzed into a buffer containing 2 mM DTT, 10 mM phosphate buffer at pH 7.2 over 24 h at 4 °C. The reassembled peptides were visibly green. Moreover, the λ_{max} for the fluorescence excitation and emission spectra were identical to that of the parent GFP (Figure 2 inset).[10] To verify that the reassembly was indeed guided by the antiparallel leucine zippers, control experiments were done with fragments with and without the leucine zippers. We found that solutions containing NGFP, CGFP, NGFP/CGFP, NZGFP/CGFP, or NGFP/CZGFP did not fluoresce, even at concentrations of over 100 μM. The apparent dissociation constant, K_{dapp}, for the NZGFP/CZGFP complex was determined by titrating NZGFP into a solution of CZGFP and monitoring the fluorescence emission intensity at 505 nm (Figure 2). The data were fit to a two-state binding isotherm, yielding a K_{dapp} of 31 $\pm$ 7 nM, and α-analysis of the binding data verified the expected 1:1 stoichiometry of NZGFP and CZGFP.[12]

Having established that GFP could be reassembled from the helix-tagged fragments in vitro, we wished to test our strategy in vivo. To this end we transformed BL21(DE3) *Escherichia coli* cells with equimolar amounts of NZGFP- and CZGFP-encoding plasmids and monitored the appearance of green color, which would identify cotransformed colonies expressing reassembled GFP. After 36 h several of the colonies turned green as illustrated in Figure 3a. with a cotransformation efficiency of 4%. Individual colonies were cultured in liquid media, and their protein expression pattern was analyzed. The green colonies were shown to express similar amounts of NZGFP and CZGFP (Figure 3b and c), whereas nonfluorescent colonies were shown to contain either

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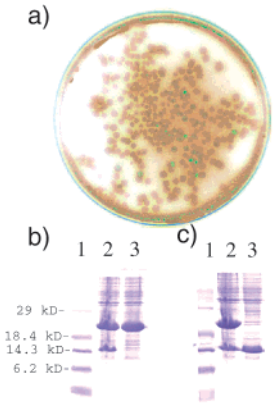

Figure 3. In vitro reconstitution of GFP demonstrated by (a) green fluorescent BL21(DE3) cells and the corresponding SDS gels of (b) lane 1: MW markers; lane 2: protein from cotransformed green colony; and lane 3: protein from colony containing only NZGFP plasmid and (c) lane 1: MW markers; protein from cotransformed green colony; and lane 3: protein from colony containing only CZGFP plasmid.

NZGFP or CZGFP. Furthermore, control cotransformation experiments with NGFP/CGFP, NGFP/CZGFP, and NZGFP/CGFP failed to show any green colonies, thus emphasizing the requirement for the presence of both NZ and CZ leucine zippers to mediate GFP assembly in vivo and in vitro.

The ability to reconstitute GFP from its peptide fragments can be extended to an in vivo fragment complementation assay for the selection of antiparallel leucine zippers as has been demonstrated for parallel leucine zippers with DHFR.[7c] It is also conceivable, that the fragmented GFP can be used to study the in vivo interaction of protein−protein pairs which have their N- and C-termini in close proximity.[7a] More generally, our protein reassembly strategy can have applications such as the selective isotopic labeling of one fragment of a large protein for NMR analysis, or the mutagenesis of a limited region of a protein as demonstrated for inteins.[13] Finally, it is also possible to envision the engineering of an on/off switch for the activity of fragmented proteins by designing a leucine zipper heterodimer which can be reversibly assembled or disassembled by controlling the environmental conditions.[14] All of these potential applications point to the widespread use of this strategy in the design and analysis of proteins.

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Supporting Information Available: Experimental details, peptide sequences and α-analysis (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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