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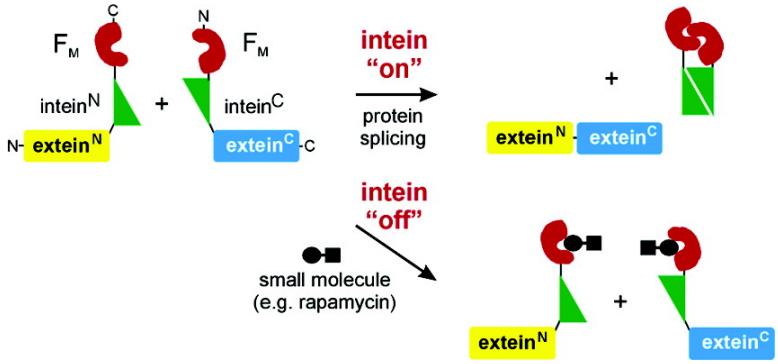

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Design of an Intein that Can Be Inhibited with a Small Molecule Ligand

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Protein splicing is a process in which an intervening sequence, the intein, catalyzes its own excision out of a larger polypeptide precursor by joining the flanking sequences, the exteins, with a native peptide bond.[1] Inteins are almost completely promiscuous toward the nature of their extein sequences. Therefore, they can serve as powerful tools for various protein engineering approaches when fused to or inserted into a heterologous protein of interest.[2] In particular, the intein-mediated formation of a peptide bond between two polypeptides offers great potential to modulate protein structure and, hence, protein function on the post-translational level, both for in vitro and in vivo applications. However, all naturally occurring inteins seem to be unregulated and spontaneously undergo protein splicing. Artificial inteins were recently reported that are dependent on a small molecule ligand.[3] Such conditionally regulated inteins can be used to control the structure and function of virtually any protein with high temporal resolution, specificity, and dosability, and by always employing the same ligand. In this work, we report the design of an intein that can be *inhibited* by the addition of a specific small molecule ligand.

Our strategy to design a ligand-inhibited intein involved the generation of a trans-splicing intein. In protein trans-splicing, the intein domain is split into two-halves that are located on two separate polypeptides, each joined with the respective N- or C-terminal extein.[1,4] The *Sce* VMA intein was artificially split into the N- and C-terminal halves that are inactive for protein trans-splicing.[3a] To turn these fragments into an active intein with an incorporated "off" switch, each was fused at its newly created terminus with the F36M mutant of FKBP12, referred to as the F_M domain (Figure 1). The F36M substitution was reported to effect a homodimerization of the usually monomeric FKBP12 protein; however, addition of the small molecule ligand, rapamycin, or synthetic derivatives thereof leads to a dissociation of the dimer.[5] We envisaged to exploit this phenomenon by first inducing close proximity of the intein halves on the basis of F_M domain dimerization, which was expected to lead to the reconstitution of the active intein. Second, addition of the small molecule ligand should prevent formation of the active intein complex and thus inhibit protein trans-splicing.

Two sets of constructs were prepared that only differed in the number of fused F_M domains (Figure 1). In constructs **1** and **2**, each intein half was fused on the genetic level to a tandem arrangement of two F_M domains. In constructs **3** and **4**, only a single F_M domain was used. Model exteins were the maltose-binding protein (MBP) as the N-terminal extein and a hexahistidine tag (His_6) as the C-terminal extein. Additional fusions with MBP and His_6 at the other end of each construct were created to allow simple protein purification by dual affinity tags (not shown in Figure 1). All constructs were separately expressed in *Escherichia coli* and purified from the soluble fraction. Protein splicing assays were carried out by mixing complementary constructs in a stoichiometric ratio at a concentration of 2 μM in the absence or presence of a small molecule ligand. The splicing reactions were monitored by densitometric analysis of Coomassie-stained SDS−PAGE gels.

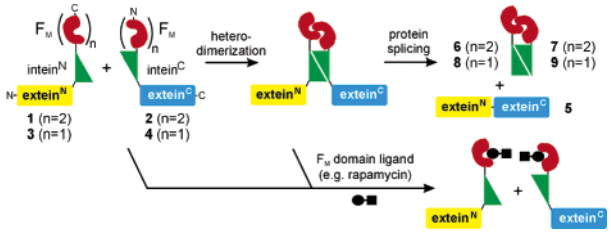

Figure 1. General principle of the ligand-inhibited intein. All constructs contained MBP and a His_6 tag, respectively, as the N- and C-terminal extein sequences. Calculated molecular masses in kDa are 89.8 (**1**), 76.6 (**2**), 77.8 (**3**), 64.6 (**4**), 45.2 (**5**), 46.6 (**6**), 74.7 (**7**), 34.5 (**8**), and 62.7 (**9**).

When **1** and **2** were incubated for 2 h at 25 °C without ligand, protein trans-splicing was evident by the formation of the three expected splicing products, namely, the joined extein sequences MBP−His_6 (**5**) and the remaining intein fusion fragments **6** and **7** (see Figures 1 and 2A). Incubation of constructs **3** and **4** under the same conditions also resulted in protein trans-splicing, yielding products **5**, **8**, and **9** (Figure 2A), indicating that the lower affinity interaction mediated by only a single F_M domain is sufficient to reconstitute the active intein (K_d of an F_M−F_M dimer is ~30 μM).[5] Protein splicing proceeded to about 39 and 44% completion for **1** and **2** and **3** and **4**, respectively, after 2 h, which are typical values also observed for other trans-splicing inteins reconstituted in vitro.[3a,b,6] MALDI-MS experiments further confirmed the identity of the splicing products.[7] In a control experiment, a derivative of construct **3**, which contained a wild-type FKBP12 instead of the F_M domain, was incubated with **4** and found not to promote protein trans-splicing.[7] These results were clear evidence that splicing is mediated by F_M domain dimerization between complementary constructs. Sufficient subunit exchange between homo- and heterodimers was obvious for both sets of constructs.

Figure 2B shows the kinetics of the splicing reactions between **1** and **2** and **3** and **4**, determined by measuring time courses over 2 h in the absence of ligands at the temperature optimum of 25 °C. Interestingly, the pair of **3** and **4** spliced significantly faster than **1** and **2** (e.g., after 20 min, the reactions went to 38% compared to 18% completion, respectively). Since the tandem F_M domains of **1** and **2** lead to a higher affinity interaction through their bivalency, this result suggests that the exchange between splicing-incompetent homodimers and splicing-competent heterodimers is the rate-determining step. Therefore, we could not assume a simple first-order reaction for protein splicing to calculate rate constants. Initial progress of the splicing reactions was about 1.4%/min for **1** and **2** and 3.2%/min for **3** and **4**. Strikingly, these rates are up to 3-fold higher than those previously reported for an artificial trans-splicing intein[3a] and up to 25-fold higher than that described for the only naturally occurring split intein, the *Ssp* DnaE intein.[6b]

To investigate the effect of ligand, dose−response curves were recorded by measuring the amount of splicing that had occurred

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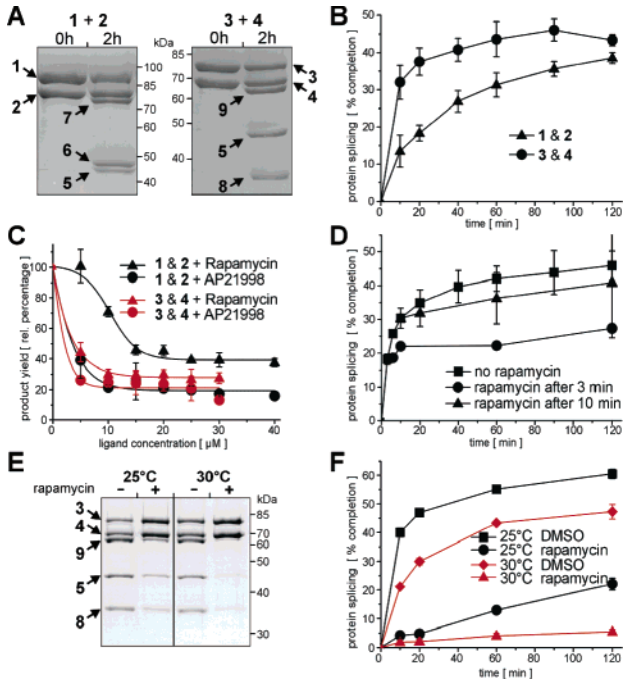

Figure 2. (A−C) Comparison of the two trans-splicing intein pairs with either two (**1** and **2**) or one (**3** and **4**) F_M domain(s) fused to each intein half: Coomassie-stained SDS−PAGE gels (A), time courses (B), and dose−response curves of ligand inhibition at the 2 h time point (C) of protein trans-splicing reactions. (D−F) Ligand-inhibition of **3** and **4**: addition of rapamycin (12.5 μM) at later time points (D). Coomassie-stained SDS−PAGE gel of the 2 h time point (E), and time courses (F) of reactions carried out at different temperatures. Reactions were performed at 25 °C or as indicated in 50 mM Tris/HCl pH 7.0, 300 mM NaCl, 1 mM EDTA, 10% (v/v) glycerine, and 2 mM DTT. Protein concentrations were 2 μM in each; rapamycin was added at 12.5 μM or as indicated. All experiments were performed, at least, in duplicate, and error bars represent standard deviations.

after 2 h at 25 °C. Indeed, both rapamycin and the synthetic ligand, AP21998 (ref 5), suppressed protein splicing, indicating that blocking F_M domain dimerization prevents formation of the active intein (Figure 2C). Saturation of inhibition was reached at ligand concentrations of about 10−15 μM for constructs **1** and **2** and at about 5−10 μM for constructs **3** and **4**. These differences correlate with the necessary stoichiometric excess of ligand to occupy all binding sites at the F_M domains (protein concentrations were 2 μM in each). Protein splicing was, in both cases, slightly more efficiently suppressed by AP21998 than by rapamycin, in agreement with the higher affinity of AP21998 for the F_M domain (K_d = ~1 nM; about 3−10-fold lower than the value for rapamycin[5]). The levels of inhibition corresponded to about 20% (AP21998) and 30−38% (rapamycin) of the product yield obtained in the absence of ligand. Addition of the corresponding amounts of the solvents DMSO and ethanol in control reactions had only a marginal effect (data not shown).

Addition of rapamycin (12.5 μM) at different time points after mixing **3** and **4** showed that already started splicing reactions could also be inhibited (Figure 2D), either by preventing further formation of splicing-competent heterodimers and/or by dissociation of heterodimers that had already formed but not yet spliced. Protein splicing still proceeded slightly after adding the small molecule (Figure 2D), indicating that the intein is not completely turned off under these conditions. This finding is consistent with the remaining splicing activity described above at ligand saturation in the dose−response curves (Figure 2C). Such a basal splicing activity at 25 °C was also previously observed for the split *Sce* VMA intein in a different context,[3b] likely due to residual affinity between the intein halves. However, this was completely alleviated when both halves were coexpressed in cells at 37 °C.[3b] We, therefore, hypothesized that residual affinity between the intein halves can be overcome at higher temperatures, probably by more pronounced unfolding of the intein fragments. Indeed, as shown in Figure 2E,F for the pair of **3** and **4**, increasing the temperature from 25 to 30 °C allowed almost complete ligand inhibition of the intein using rapamycin. The ratio of the splicing yield between uninhibited and ligand-inhibited intein was significantly improved at the higher temperature (from ~3-fold at 25 °C to ~10-fold at 30 °C after 2 h). A control experiment involved replacement of the F_M domain of **4** with an FRB domain (construct **4a**) that cannot dimerize with the F_M domain of **3**. Incubation of **3** and **4a** showed a comparable basal intein activity at 25 °C that could be suppressed at 30 °C.[7] This finding further substantiates the hypothesis that this basal activity is due to, at least in large part, residual affinity between the intein halves. Future studies will investigate the in vivo performance of the new ligand-inhibited inteins, for example, in yeast or mammalian cells. Ligand-controlled F_M domains have been previously used in living cells to control protein aggregation.[5,8]

In summary, the activity of a split protein was controlled by a cell-permeable small molecule that acts as an inhibitor of protein fragment complementation. This new concept to incorporate an off switch into proteins was applied to the design of a conditional trans-splicing intein that can be inhibited in a dose-dependent manner. Coupling this general tool to proteins of interest should allow highly specific control of protein structure and function on the post-translational level. This approach may represent an attractive alternative to common genetic approaches such as conditional promoters and RNAi techniques.

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Supporting Information Available: Full experimental procedures and MS data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Noren, C. J.; Wang, J.; Perler, F. B. *Angew. Chem., Int. Ed.* **2000**, *39*, 450−466. (b) Paulus, H. *Annu. Rev. Biochem.* **2000**, *69*, 447−496.
(2) For example, see: (a) Muir, T. W.; Sondhi, D.; Cole, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6705−6710. (b) Evans, T. C., Jr.; Benner, J.; Xu, M. Q. *Protein Sci.* **1998**, *7*, 2256−2264. (c) Chin, H. G.; Kim, G. D.; Marin, I.; Mersha, F.; Evans, T. C., Jr.; Chen, L.; Xu, M. Q.; Pradhan, S. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 4510−4515. (d) Kim, S. B.; Ozawa, T.; Watanabe, S.; Umezawa, Y. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 11542−11547. (e) Zeidler, M. P.; Tan, C.; Bellaiche, Y.; Cherry, S.; Hader, S.; Gayko, U.; Perrimon, N. *Nat. Biotechnol.* **2004**, *22*, 871−876.
(3) (a) Mootz, H. D.; Muir, T. W. *J. Am. Chem. Soc.* **2002**, *124*, 9044−9045. (b) Mootz, H. D.; Blum, E. S.; Tyszkiewicz, A. B.; Muir, T. W. *J. Am. Chem. Soc.* **2003**, *125*, 10561−10569. (c) Buskirk, A. R.; Ong, Y. C.; Gartner, Z. J.; Liu, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 10505−10510. (d) Mootz, H. D.; Blum, E. S.; Muir, T. W. *Angew. Chem., Int. Ed.* **2004**, *43*, 5189−5192.
(4) Wu, H.; Hu, Z.; Liu, X. Q. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9226−9231.
(5) Clackson, T. et al. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7096−7101.
(6) (a) Lew, B. M.; Mills, K. V.; Paulus, H. *Biopolymers* **1999**, *51*, 355−362. (b) Martin, D. D.; Xu, M. Q.; Evans, T. C., Jr. *Biochemistry* **2001**, *40*, 1393−1402.
(7) See Supporting Information for full experimental details.
(8) Rivera, V. M. et al. *Science* **2000**, *287*, 826−830.

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