enhanced activity but destabilized the enzyme, and was not incorporated into the final mutant OB-1. There are 15 mutations in OB-1, including five (two synonymous) in the α-factor leader sequence, that collectively contribute to both the expression/secretion level and specific activity while retaining wild-type thermostability. As such, mutant OB-1 is more thermally stable than some native thermostable fungal laccases and also highly resistant to organic solvents and acidity, making it an excellent candidate for industrial applications. In addition, the general nature of the mutant leader peptide is likely to enhance secretion levels when used with other proteins.

Laboratory evolution of highly active enzymes tends to cause the engineered enzymes to lose activity toward substrates not used during screening, a consequence that may result from the altered shapes of enzyme active sites. To maintain the broad substrate specificity of PM1 laccase, Maté et al. (2010) employed two representative substrates in their screen. While such a strategy is not always effective in maintaining broad substrate specificity, the resulting mutant OB-1 exhibits increased specific activity toward all three substrates examined with only a slight preference for ABTS rather than guaiacol when compared with the third-generation mutant. In some applications, such as delignification, a chemical mediator is usually used to indirectly oxidize phenol and nonphenol compounds (Call and Mucke, 1997). As such, a highly active laccase toward a chemical mediator (for example, 1-hydroxybenzotriazole for delignification) would eliminate the necessity of maintaining broad substrate specificity in laccase-directed evolution. In addition, the inadvertent introduction of destabilizing mutation F454S could also have been circumvented if a thermal stability screen were incorporated into the initial library screening, though such a screen may not have been feasible in the presence of poor total enzymatic activity. Nevertheless, this fine piece of work represents another interesting example of how directed evolution and rational enzyme design can be combined for the engineering of protein function, and how such a strategy could enhance the efficiency of searching protein sequence space (Arnold, 2006; Turner, 2009). More immediately, Maté et al. (2010) have developed a heterologous expression system for efficiently producing HRPLs, which has been a major hurdle for industrial applications of laccases, and the highly active and stable OB-1 is an excellent starting point for various biotechnological applications of HRPLs.

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A Group I Intron Riboswitch

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DOI 10.1016/j.chembiol.2010.09.001

Riboswitches are mRNA-based elements that regulate gene expression via binding of a specific ligand. Breaker and coworkers have now discovered a novel type of regulatory RNA motif that acts by c-di-GMP-dependent control of a self-splicing group I intron ribozyme (Lee et al., 2010).

Riboswitches are widespread regulatory motifs found in mRNAs. They are usually composed of an aptamer domain facilitating ligand binding and a second domain termed an expression platform (Roth and Breaker, 2009). The latter reorganizes in response to ligand binding to the aptamer domain, resulting in altered gene expression. Commonly one out of two different mechanisms is employed: either the formation of a Rho-independent termination structure is regulated, resulting in the premature termination of transcription, or the formation of a stem-loop structure that masks the ribosome binding site is controlled, resulting in the regulation of translation initiation. Exceptions are represented by riboswitches operating in eukarya, as well as an autocatalytic motif (the *glmS* riboswitch in certain eubacteria) that cleaves the mRNA in response to glucosamine-6-phosphate binding (Winkler et al., 2004).

Now Breaker and coworkers have described a novel type of riboswitch that contains a self-splicing ribozyme as an expression platform (Lee et al., 2010).

The riboswitch was discovered during the investigation of a new aptamer motif identified by bioinformatics approaches (Weinberg et al., 2010). The novel motif binds the bacterial second messenger 3′-5′-cyclic-di-guanylic acid (c-di-GMP), responsible for signaling microbial processes such as biofilm formation, virulence, and motility. This aptamer differs from the first class of c-di-GMP aptamers present in recently discovered riboswitches (Sudarsan et al., 2008). The novel motif (termed c-di-GMP-II) is frequently found in *Clostridia* and some other eubacteria. Predominantly, these c-di-GMP-II motifs are connected to an expression platform controlling the termination of transcription. One motif, however, was unusually distant (about 600 nt upstream) from the open reading frame (ORF). When they looked closer, Breaker and coworkers discovered that this stretch of RNA residing between the c-di-GMP-II aptamer and the ORF had all of the characteristics of a group I intron ribozyme.

Group I introns catalyze a self-splicing reaction by binding guanosine or a corresponding nucleotide that then attacks a 5′-splice site (5′-SS). Next, the resulting 5′-exon attacks at the 3′-SS, generating the exon-exon splice product and the free intron containing the ribozyme. Within group I introns, protein-coding ORFs are often found. For example, transposases are frequently associated with self-splicing ribozymes. The combination of both activities generates a mobile selfish genetic element that is able to insert into RNAs without doing much harm to the host genome (Haugen et al., 2005). In *Clostridium difficile*, nine out of ten group I introns are found in such a context. On the other hand, the intron that carries a c-di-GMP-II aptamer upstream is not associated with a transposase gene, pointing at the possibility that a locally defined function might be attributable to the ribozyme at this position.

By a series of sophisticated experiments, Breaker and coworkers examined the reactivity of the c-di-GMP aptamer/group I intron combination. It seems that the intact, full-length mRNA is not translated because the start codon is sequestered by interactions with a part of the intron sequence. The second messenger binding to the c-di-GMP-II aptamer controls where the ribozyme-mediated initial attack of a guanosine triphosphate (GTP) occurs. In absence of c-di-GMP, the mRNA is cleaved just 4 nt upstream of the start codon, resulting in an mRNA that is not translated due to the absence of a ribosome-binding site. An alternate site is attacked and splicing occurs only in the presence of c-di-GMP, assembling an efficient ribosome binding site as well as cutting out the intron ribozyme together with the sequence that masked the start codon. Hence, it is proposed that a functional message that can be translated is generated by the ligand-controlled ribozyme clipping itself from the mRNA.

The discovery by the Breaker group reveals how nature combines distinct RNA functions such as ligand binding and catalytic activity in a highly modular fashion. An artificial system that exploits the same modularity logic and is composed of a theophylline-regulated group I intron was described previously by Ellington and coworkers (Thompson et al., 2002). It is fascinating to observe that life actually makes use of mechanisms that researchers have designed and constructed in order to prove the versatility of RNA for such purposes. However, one could wonder why such a "noneconomic" way of control is implemented, since the group I intron is a rather large element compared with the frequently used expression platforms in riboswitches described above. As evidenced by several examples of c-di-GMP-dependent riboswitches with at least two different classes of aptamers, there are more nucleotide-efficient ways of controlling gene expression by this second messenger.

On the other hand, the discovered allosteric group I ribozyme possesses a further property that is absent in other c-di-GMP-sensing elements: since the group I intron requires GTP as cofactor for the self-splicing reaction, the riboswitch may be regarded as a tandem switch, sensing both GTP and c-di-GMP simultaneously. In the presence of GTP and c-di-GMP, an RNA product that gets translated is produced, resembling a Boolean logic AND gate. Such tandem riboswitches have been discovered before where two distinct RNA regulators act in concert in one 5′-untranslated region (Sudarsan et al., 2006). Further studies, also with respect to the function of the gene product under control of the novel c-di-GMP-II riboswitch that is currently annotated as a putative surface protein, will be necessary in order to clarify whether a potential tandem property of the switch is indeed useful in this context.

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