# DNA Detection and Signal Amplification via an Engineered Allosteric Enzyme

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We describe the design, synthesis, and functional characterization of an intrasterically regulated semisynthetic enzyme and its application in sequence-specific DNA detection.[1,2] The system is composed of covalently associated *i*nhibitor-*D*NA-*e*nzyme (IDE) modules and functions via DNA hybridization-triggered allosteric enzyme activation and signal amplification through substrate turnover.[3] Its functional capacity is highlighted by the sequence specific detection of approximately 10 fmol DNA in less than 3 min under physiological conditions. These studies suggest that rationally designed intrasterically regulated enzymes may constitute a promising new class of reagents for highly sensitive, rapid, and PCR-independent one-step detection of label-free DNA sequences.

The principles that govern intrasteric regulation in natural enzymes[4] are firmly rooted in the physicochemical advantages of intramolecular binding. These enzymes often use an appending N- or C-terminal polypeptide pseudosubstrate to block access to their active sites. Enzyme regulation (activation) occurs at an allosteric site, typically the site between the enzyme and pseudosubstrate, where a conformational change or cleavage event removes the pseudosubstrate from the active site. We sought to mimic the intrasteric regulatory features of natural enzymes by designing novel semisynthetic constructs that can be activated in the presence of complementary DNA sequences. The IDE complex described employs a single-stranded DNA (ss-DNA) probe to covalently tether Cereus neutral protease[5] (CNP) to its small-molecule phosphoramidite inhibitor.[6] We anticipated that the conformational flexibility of the ss-DNA tether in an appropriately functionalized system would allow facile intramolecular binding of the inhibitor to the enzyme active site to furnish the *inactive state* of the IDE (Figure 1). Likewise, we postulated that IDE should rapidly convert to an active state in the presence of its complementary DNA sequence because formation of a high-affinity DNA duplex structure was expected to drastically alter the conformation of the tether and favor the liberation of the inhibitor from the enzyme active site. Once activated, the enzyme would act upon its fluorogenic substrate, present in situ, producing an optical output signal. Moreover, because the enzyme turns over many copies of the substrate, in principle each probe hybridization event can be chemically amplified to furnish high detection sensitivity.

The foremost variable in the design of an IDE complex is the enzyme module, which must be compatible with the allosteric DNA tether and possess high intrinsic catalytic activity to afford rapid rates of signal evolution. The choice of the enzyme in turn dictates the type of inhibitor and substrate to be used. In the present design we have employed CNP,[5] an endolytic extracellular zinc metalloprotease from *Bacillus cereus*, as the enzyme constituent of the IDE. Its high structural homology to thermolysin[7] was invaluable in selecting the inhibitor module[6] and the inhibitor−DNA and DNA−enzyme attachment sites used in our design (vide infra). Molecular modeling was used to determine the attachment sites that seemed to minimize DNA strain and unfavorable steric inter-
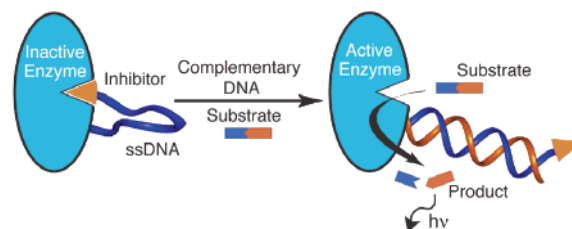


**Figure 1.** Schematic representation of an intrasterically inactivated *i*nhibitor-*D*NA-*e*nzyme (IDE) construct (left) and the DNA hybridization-triggered enzyme activation (right). IDE can be used to sense low concentrations of complementary DNA because of its built-in capacity for signal amplification via rapid substrate turnover.

actions in the intramolecular binding of the inhibitor to the enzyme active site. On the basis of the modeling analyses, the surface-exposed glutamic acid 151 of CNP was chosen as the DNA−enzyme ligation site. Furthermore, because the wild-type CNP lacks any cysteine residues, the $CNP_{E151C}$ mutant was chosen as our target enzyme to afford a chemoselective handle for DNA attachment. The CNP gene[8] was amplified by PCR using genomic DNA from *B. cereus* (strain DSM3101) as the template. The resulting gene, which coded for the proenzyme of cereus neutral protease, was then mutated (E151C) by using an overlap extension-PCR method[9] and inserted into pET19b (Novagen) to afford the expression plasmid pCNP151C. $CNP_{E151C}$ was overexpressed in *Escherichia coli* BL21(DE3) (Novagen), isolated as inclusion bodies, refolded, and purified by D-phenylalanine affinity chromatography.

The enzyme substrate, DABCYL-$\beta$Ala-Ala-Gly-Leu-Ala-$\beta$Ala-EDANS, was designed on the basis of CNP's substrate selectivity and prepared in two steps by the standard solid- and solution-phase methods.[10] Protease activity was measured as a function of increasing EDANS fluorescence in time as the result of the endolytic cleavage of the peptide substrate separating the EDANS fluorophore from the DABCYL quencher. The measured $k_{cat}$ and $K_m$ values of 165 s$^{-1}$ and 15 $\mu$M, respectively (20 mM Tris, pH 7.0), are in good agreement with literature values for the wild-type protease.

Naturally occurring inhibitors of this class of proteases often utilize the phosphoramidite as the transition-state mimic.[6] By adopting this class of inhibitors to our IDE design, we were also able to develop a convenient solid-phase synthetic process for the production of the desired inhibitor−DNA conjugate (Scheme 1).
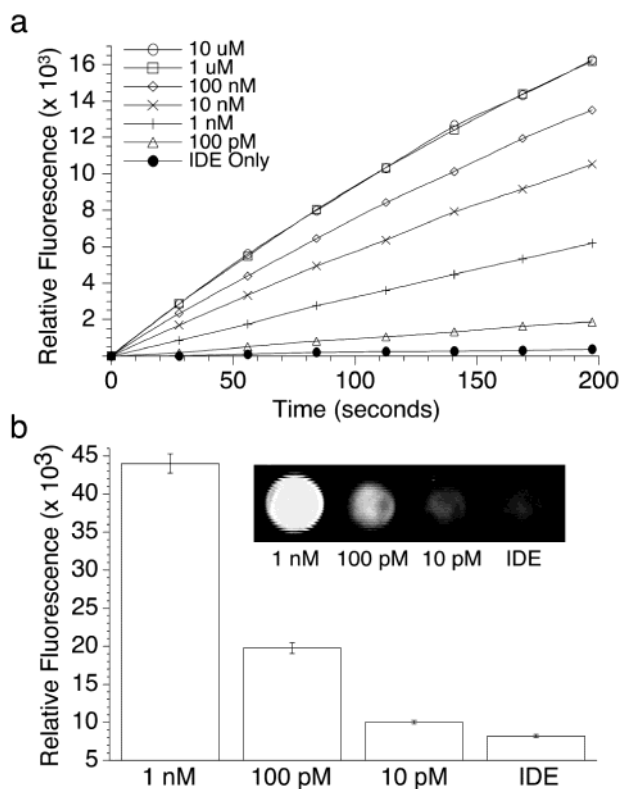
*Scheme 1*

## a



## b
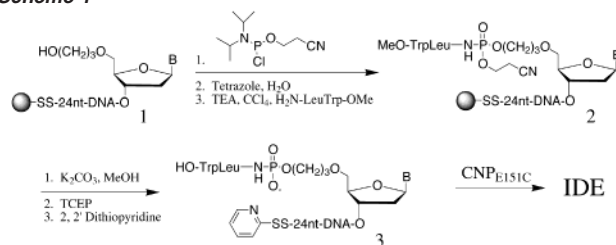


**Figure 2.** Effect of complementary DNA concentration on IDE activity. (a) Activity was measured through endolytic cleavage of substrate (40 mM) in the presence of 1.5 nM IDE (1 M NaCl, 20 mM TRIS, pH 8) using a fluorescent plate reader ($\lambda_{ex} = 360$, $\lambda_{em} = 465$). (b) Fluorescence measurements 80 min after addition of substrate showed a lower detection limit of 10 pM complementary DNA. Additionally, IDE activity could be visualized using a broad-band UV lamp and CCD detector (inset), which has important practical applications for high-throughput screening.

The support-bound 24-mer oligonucleotide[11] **1** having a 3′-thiol-modifier C3 S−S CPG and a 5′-C3 spacer was synthesized according to the reagents and procedures recommended for ultramild DNA synthesis (Glen Research). Subsequent reaction with 2-cyanoethyl diisopropyl chlorophosphoramidite followed by oxidation afforded the corresponding 5′-H-phosphonate diester intermediate. The H-phosphonate was then reacted with H₂N-LeuTrp-OMe in the presence of triethylamine and carbon tetrachloride[12] to give the protected phosphoramidite-containing DNA-phosphoryl-LeuTrp-OMe **2**. The DNA construct was then deprotected in aqueous MeOH/K₂CO₃ and cleaved from the solid support by disulfide reduction in the presence of tris(2-carboxyethyl) phosphine (TCEP). The free 3′-thiol moiety was then activated as a pyridyl disulfide, and the resulting oligonucleotide-inhibitor complex **3** was purified by polyacrylamide gel electrophoresis and RP-HPLC and characterized by MALDI-MS. The coupling of inhibitor−DNA conjugate **3** to CNP$_{E151C}$ took place smoothly and in high yields under slightly basic conditions. The desired IDE complex was purified to homogeneity by anion-exchange chromatography (FPLC) and further characterized by gel electrophoresis.

IDE was found to be remarkably sensitive in detecting low concentrations of complementary DNA. The correlation between

IDE activity and complementary DNA concentration (10 $\mu$M to 10 pM) was examined by equilibrating complementary DNA with IDE (1.5 nM) for 5 min prior to addition of 40 $\mu$M substrate (Figure 2a). Even at 100 pM complementary DNA concentrations (10 fmol), a 5-fold increase in initial rate over IDE alone was observed, giving rise to a detectable signal in less than 3 min. Furthermore, 10 pM DNA concentrations could be distinguished over the background signal if the assay was extended to 80 min (Figure 2b). Conversely, IDE could not be activated in the presence of noncomplementary DNA[11] even at the highest tested concentration (10 $\mu$M). Moreover, noncovalently associated ID−enzyme complexes did not display increased protease activity in the presence of complementary DNA, further supporting the conformational and the covalent anchoring requirements of the intrasterically regulated IDE.

The above experiments highlight the potential of IDE in practical applications where sensitivity and simple detection methods are a requirement.

**Supporting Information Available:** Cloning and expression of CNPE151C and synthesis and purification of IDE and substrate (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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