Encoded reaction cassette for the highly sensitive detection of the making and breaking of chemical bonds

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ABSTRACT A reaction cassette has been designed for the highly sensitive detection of the making and breaking of chemical bonds. The system is envisioned as a companion device to be used in the search for antibody and other novel catalysts. The cassette also may have important clinical applications in the design of diagnostic reagents. In its fully encoded format, this methodology is capable of both detecting and decoding chemical events.

The essence of antibody catalysis is the creation of a library of molecules from which those with unique chemical potential are selected. As for any application of combinatorial chemistry, the successful identification of useful molecules depends not only on the chemical insight that was used to create the library but also on the ease and sensitivity by which the library can be screened (1). In essence, the problem reduces to the separate issues of display and detection. In the case of antibodies, the conversion of diversity into combinatorial libraries in phage where the recognition and replication functions are linked in a single entity has allowed simple binding events to be monitored, thus solving the display problem. However, the problem of the direct detection of antibody catalysts at the level of phage particles remains (2, 3). To accomplish this, one needs to detect catalysis at the level of only a few molecules (4, 5) either by use of selection or highly sensitive assays (6). Here, we report success with a concept wherein a reaction cassette is constructed that should be capable of detecting the making or breaking of any chemical bond, even when only a few molecules of product are formed. In principle, the cassette can be considered to be a companion assay device that can be constructed for any system in which a new catalyst or reagent is sought.

MATERIALS AND METHODS

Substrate Synthesis. The peptide was assembled manually on Tentagel support (Novabiochem or Rapp Polymere) according to standard fluorenylmethoxycarbonyl (Fmoc) methodology (7). To have a final loading of 40–60 μmol of peptide per g of support, the first step of the synthesis was performed with a 4-fold excess of solid support to Fmoc amino acids; after all final washings, the unreacted amino groups were capped by treating twice for 10 min each with 0.25 volume of 4.23 M acetic anhydride in 2,6-lutidine/0.75 volume of 0.53 M N,N-dimethylaminopyridine in tetrahydrofuran (THF). The overall yield for peptide synthesis was ~98%.

The tert-butyl (t-Bu) protecting group for the hydroxyl moiety on tyrosine was removed by treatment with 5% (vol/vol) ethanediithiol in anhydrous CF3COOH for 2 h, followed by extensive washing with dichloromethane (DCM), methanol, and N,N-dimethylformamide. The Fmoc protecting group was removed before coupling to the linker L-1 (4-(O-dimethoxytrityl)hydroxybutyrate, sodium salt; see Fig. 3). The matrix containing tyrosine-O-L-1 was converted to tyrosine-O-acetyl after selective deprotection of the phenolic ring (by treatment with concentrated NH4OH for 3 h) and capping (treatment with 0.25 volume of 4.23 M acetic anhydride, in 2,6-lutidine/0.75 volume of 0.53 M N,N-dimethylaminopyridine in THF for 30 min). The yield after each step was determined by the dimethoxytrityl cation assay (8).

Linker L-1 was prepared in one step from the sodium salt of 4-hydroxybutyrate and dimethoxytrityl chloride in pyridine (9).

DNA Synthesis. DNA synthesis was carried out on a 394 Applied Biosystems DNA synthesizer using standard phosphoramidite chemistry (10). The standard 1 μmol cycle was modified as follows: (i) Washing steps 3, 5, 61, 66, 77, and 94 were prolonged to 30 s (the use of longer or shorter times decreased the yield); (ii) the incubation time with phosphoramidite and tetrazole (step 45) was prolonged from 25 s to 120 s; and (iii) the concentration of the phosphoramidites was increased from 0.1 M to 0.2 M. Under these conditions, the average yield per step was ~97%. The bases were deprotected upon treatment with concentrated NH4OH for 20 h at 55°C. The dimethoxytrityl group is removed upon treatment with 3% CCl4/CH3OH in DCM for 5 min, followed by extensive washing with DCM, THF, methanol, 20 mM Tris-HCl, pH 8/160 mM NaCl, and distilled water (dH2O). After this step, the cassette is ready for use.

Enzymatic Cleavage and Inhibition Experiments. The cassette (1 mg; 5.9 μmol of substrate peptide–polynucleotide hybrid per g of solid support) was suspended in 20 μl of 20 mM Tris-HCl, pH 8/160 mM NaCl and 170 μl of dH2O. 0.85 nmol of trypsin, pepsin, carboxypeptidase A, a-chymotrypsin, or a-chymotrypsin and 1 mg of Bowman–Birk inhibitor (11) in 10 μl of dH2O was added to the reaction medium, and the mixture was shaken at 20°C. Supernatant fluids (18.7 μl) were taken after 30 min and were submitted to the PCR.

PCR Experiments. Aliquots (18.7 μl) from the reaction mixture were mixed with the following PCR components (Promega): 1.2 μl of 2.5 mM MgCl2, 2 μl of Taq buffer, 1.6 μl of 2.5 mM deoxynucleotide triphosphates, and 1 μl of primers at 100 pmol/μl. Taq polymerase (2.5 units; 0.5 μl) was added just before starting the first PCR cycle. A positive control (PCR components only) was run with dH2O containing 1 pmol of the polynucleotide sequence used in this study. A negative control was run under the same conditions without the polynucleotide sequence. The PCR was run on a Perkin–Elmer/Cetus 9600 instrument with the following cycle program: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. After 35 cycles the results were analyzed on agarose gels [1% Gibco/BRL agarose/2% FMC NuSieve GTG agarose with 90 mM Tris/64.6 mM borate/2.5 mM EDTA, pH 8.3 (1 X TBE) at 103 mV].

Fluorescence Assay. After the PCR, the reaction medium (25 μl) was transferred to a 96-well ELISA plate and diluted

Abbreviations: Fmoc, fluorenylmethoxycarbonyl; DCM, dichloromethane; THF, tetrahydrofuran; dH2O, distilled water; t-Bu, tert-butyl.

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to 250 μl with dH2O (175 μl) and methanol (50 μl). The probe (1 μl; YOYO-1, Molecular Probes) was added to this medium, and the results were analyzed under UV light at 254 nm.

Uncatalyzed Reactions. The cassette lacking the substrate unit was prepared as follows: TentaGel bearing a hydroxyl group functionality (1 g) was shaken with 10 equivalents (85 mg) of dimethoxytrityl chloride in 4 ml of pyridine at room temperature for 3 days. Titration of the dimethoxytrityl group showed a loading of 32 μmol/g of solid support. The unreacted hydroxyl groups were acetylated for 30 min with 0.25 volume of 4.23 M acetic anhydride in 2.6-lutidine/0.75 volume of 0.53 M N,N-dimethylaminopyridine in THF. DNA synthesis was performed on this matrix by following the procedure described above.

The cassette lacking the substrate (0.5 mg; 12.2 μmol of polynucleotide per g of solid support) and the cassette with the substrate–polynucleotide hybrid (1 mg; 6.2 μmol of substrate peptide per g of solid support) were suspended separately in 20 μl of 20 mM Tris-HCl, pH 8/160 mM NaCl and 180 μl of dH2O. The mixtures were shaken at 20°C and aliquots (18.7 μl) taken after 2, 16, 29, and 51 h were subjected to the PCR.

**DESIGN FEATURES OF THE CASSETTE**

The approach combines the use of the two well-known techniques of synthesis on solid support and the PCR. (For indirect approaches to detect antibody catalysis, see refs. 12–14.)

The central operative feature of the reaction cassette is the liberation (cleavage event) or capture (bond formation) of a polynucleotide containing two primers (Fig. 1). Thus, when an appropriately functionalized solid support is exposed to a catalyst or a library of catalysts that are able to selectively cleave the reaction cassette at the substrate juncture, single-stranded DNA (polynucleotide) will be released and can be amplified by the PCR. Furthermore, the sequence of the polynucleotide may be chosen in such a way that it reflects the nature of the substrate, so that a library of encoded substrates can be designed (15, 16). When substrate libraries such as these are exposed to a library of catalysts, one can identify not only the catalyst but also the substrate, since the sequence of the cleaved polynucleotide encodes and thus identifies which substrate sequence has been cleaved. In addition, our cassette reaction technique allows one to follow a bond formation event via the inverse pathway (Fig. 1). In this initial report we describe the application of this technology to the study of enzyme-catalyzed bond cleavage.

The Matrix. In attempting to implement this technology, we encountered a variety of problems. First, certain matrix materials, like CPG (Controlled Pore Glass), were labile and liberated the polynucleotide–substrate hybrid leading to an undesired background reaction. We presumed that cleavage of a bond between the solid support and the first linker was responsible for this problem. For our purposes, TentaGel was found to have much better mechanical and chemical properties. Our strategy using this support is shown in Fig. 2.

**Synthesis of the Substrate Portion of the Cassette.** The catalyst chosen to study our cassette methodology had to be chemically and physically well defined. α-Chymotrypsin seemed to be an ideal enzyme for this purpose. The substrate portion of the cassette was (L-Ala)2-L-Tyr-(L-Ala)2, which is known to be the best substrate for α-chymotrypsin (17). The choice of this substrate was dictated by the fact that our goal in these initial studies was to explore the lower limit of the sensitivity of the system.

It was not necessary to introduce a spacer between the solid support and the substrate since the TentaGel matrix is en-
dowed with an extended polyethylene glycol arm bearing a terminal functional group that allows the direct attachment of the cassette substrate (Fig. 2). The loading of the matrix is about 280 μmol/g. To avoid the generation of hindered sites, we functionalized only the most exposed ones (40–60 μmol/g) by adding a large excess of matrix in the first step of the synthesis and capping the unreacted groups. We anticipated that the spacing between the substrate and the DNA tag was to be one of the critical design features of the cassette. As such a number of linkers have been prepared, to date linker L-1 appears to be the most appropriate (Fig. 3). (The synthesis of these linkers will be described elsewhere.)

The peptide sequence that we have chosen as a substrate requires a deprotection step on the tyrosine-O-t-Bu with 5% ethanedithiol LN anhydrous CF₃COOH for 2 h. Since we cannot perform this deprotection after the polynucleotide synthesis (concentrated acid leads to many side reactions on the polynucleotide), we had to change the tyrosine-protecting group from a t-Bu group to a base-labile protecting group that can be removed at the same time as the deprotection of the DNA. In Fig. 3 we show the pathway followed for protecting-group exchange and for the introduction of the linker. Aside from its utility for the present experiment, this synthesis shows that the system can be readily transformed and modified chemically so as to be compatible with a variety of substrates.

**Synthesis of the Polynucleotide Portion of the Cassette.** The polynucleotide is a 45-mer (3'-GAT TCT TAT CCC GGG CTG ATC GTC CTC GAG GGA ACC CTT CAT CGA-5'). It possesses two primer sequences (15 nucleotides) and one encoding sequence (15 nucleotides) that identifies the substrate, which in this case is a pentapeptide in which each amino acid is arbitrarily assigned a triplet nucleotide sequence. Any nucleotide sequence may be used to encode the nature of the substrate, and the choice of the nature of the code will depend primarily on the number and complexity of test substrates.

**Reaction Specificity.** The cassette was submitted to enzyme cleavage, and the results after the amplification by PCR of the liberated polynucleotide are shown in Fig. 4. Lanes 1–8 show the results after incubation at 20°C for 30 min with trypsin, pepsin, papain, carboxypeptidase A, proteinase K, α-chymo-
trypsin, a-chymotrypsin/Bowman–Birk inhibitor (11), or no enzyme. Lane 9 corresponds to the positive control and lane 10 to the negative control.

The data in lane 6 of Fig. 4 show that in the presence of a-chymotrypsin, a band corresponding to 45 nucleotides was present, indicating a net cleavage of the substrate by this enzyme. This interpretation was further supported by the control experiments. When the cassette was incubated with trypsin, pepsin, papain, or carboxypeptidase A, no band could be detected on the agarose gel, which is in agreement with the specificity of these enzymes. When the Bowman–Birk inhibitor was added to a-chymotrypsin (lane 7), no cleavage was detected. As expected, in the presence of proteinase K, a band was detected indicating a net cleavage by this enzyme. The intensity of the band indicates that the cleavage by proteinase K is weaker than that accomplished by a-chymotrypsin. This result is in agreement with the fact that a-chymotrypsin is specific for the substrate used in this study. In the absence of any enzyme (lane 8) no cleavage is detected after 30 min.

Under the reaction conditions used in this study, the enzymatic activity of 1 pmol of a-chymotrypsin was readily detected. One should be able to improve the sensitivity of detection since the substrate concentration (29.5 μM) used in our experiment was well below saturation (17). Additionally, preliminary experiments have shown that a longer linker between the substrate and the polynucleotide enhances the accessibility of the enzyme to the substrate (data not shown).

An alternative to analyzing the PCR products on agarose gel, which can become laborious when libraries of catalysts are being screened, one can simply add to the reaction mixture a fluorescent probe that undergoes fluorescence enhancement upon intercalation into the DNA. The insert in Fig. 4 shows a photograph taken under UV light at 254 nm of the reaction medium in the presence of the YOYO-1. The first well corresponds to the experiment in lane 6, the second well to the experiment in lane 8, and the third to the probe in buffered solution without any additives. The greatest fluorescence enhancement is in the first well, which contains the amplified DNA. The second well shows a background fluorescence resulting from the interaction of the probe with the primers. As expected, the third well does not show any detectable fluorescence. Another advantage of the YOYO-1 probe is that the amount of the PCR product (which should be directly related to the efficiency of the enzyme cleavage) can be quantified (18).

Reaction Sensitivity. It was interesting to note that after 24 h, in the absence of a-chymotrypsin, a band corresponding to the DNA 45-mer was detected (data not shown). This background reaction can be due to bond solvolysis anywhere between the solid support and the first bases of the polynucleotide or simply to a leakage from the matrix. In an attempt to define the cleavage site(s), we prepared the same cassette lacking the substrate unit (where the polynucleotide is directly linked to the matrix via a mixed phosphodiester bond). When this matrix and the standard cassette were incubated separately without any catalyst, one could detect a background reaction after 16 h only in the cassette containing the peptide unit. The ease of detection of this uncatalyzed reaction increased between 29 h and 51 h. After 29 h the background reaction was detected in the cassette lacking the peptide as well. If the uncatalyzed reaction originated from leakage from the matrix or solvolysis of a phosphodiester bond, the 45-mer DNA would have been detected at the same time irrespective of whether the cassette contained a substrate unit. The fact that detectable cleavage after 16 h was limited to cassettes containing the peptide substrate indicates that bond solvolysis occurs in the substrate sequence, most likely at a peptide bond. After 16 h, when background cleavage of the peptide bond was observed, solvolysis of the phosphodiester bond was not detected (Fig. 5).
Fig. 5. Time course of the cassette reaction. In the example shown, the PCR product is taken from the medium containing (i) the cassette lacking the substrate (lanes −) and (ii) the standard cassette (lanes +). The positive control corresponds to the PCR product of an authentic sample of the DNA 45-mer. The negative control corresponds to the same experiment without the DNA 45-mer.

However, by 29 h solvolysis of the phosphodiester bond was detected.

When one assumes that the rate constant for a peptide bond hydrolysis is $<3 \times 10^{-9} \text{ s}^{-1}$ ($t_1/2 > 7$ years) (19), at a cassette concentration of 29.5 µM the velocity for peptide bond hydrolysis is $\sim 9 \times 10^{-14} \text{ M/s}$. After 16 h one would expect to have $\sim 5 \text{ nM}$ of free polynucleotide in solution. This amount is known to be readily detectable by the PCR. Since the rate constant for phosphodiester bond hydrolysis (20) is much slower ($5.7 \times 10^{-14} \text{ s}^{-1}$) than that of peptide bond hydrolysis, the background for the cassette lacking the substrate unit will be detected only after longer incubation times.

**Practicality of the Encoded Cassette System.** We have shown that the cassette system works reproducibly and that the entire cassette can be assembled in less than 48 h by using conventional synthetic chemistry. Because of the simplicity and versatility of our technology, analysis of a large number of potential catalysts can be carried out in $<4$ h. Although the current limit of detection is $\sim 1 \text{ pmol}$ of $\alpha$-chymotrypsin, the sensitivity and efficiency of this system can be readily improved. These improvements may be achieved either by increasing the concentration of the substrate and/or its loading on the solid support and/or by introducing longer linkers between the substrate and the polynucleotide.

The system is not limited to transformations in which bond cleavage or bond formation is the initial event. The only requirement is that the chemical transformation make a bond labile to other reagents. For example, in the search for dihydroxylation catalysts (21), an olefin can be used as a substrate because when it is dihydroxylated it can be selectively cleaved by periodate. Additionally, one can envision systems in which the transformation modifies the cassette so that it becomes a substrate for a known enzyme.

Finally, even using PCR conditions that are not yet optimized, we were able to detect in a matter of hours uncatalyzed chemical reactions with half-lives of years. This result leads us to conclude that almost any catalytic bond cleavage or formation event can in principle be readily detected in a very short time. The method should be applicable to detection of events that are of low efficiency either because the enzyme is poor or, more important, because the catalyst is only one member of a large library and, thus, is present in low concentration.

**CONCLUSION**

While our system was developed for antibody catalysis, it is not limited to this and can be used for any synthetic or enzymatic reaction including those important to diagnostic assays in medicine. When one is using the cassette to search for a single reaction, only one DNA sequence is necessary. However, in a fully encoded format, one can test multiple substrates simultaneously by using unique polynucleotide sequences for each substrate. The nature of the reaction that occurred is simply determined by the sequence of the polynucleotide (22) either after the PCR reaction or upon cloning of the polynucleotide. In essence, one can create an encoded combinatorial library of substrates to learn about reaction specificities. One can imagine designing systems in which a combinatorial library of catalysts is screened against a combinatorial library of substrates to find new catalysts and refine their substrate specificity in a single operation. Finally, the ease of constructing encoded cassettes suggests an operating procedure in which a substrate cassette is built as a companion to any experiment where one is searching for a new catalyst. This frees the experimenter to design experiments that are independent of whether the reaction products can be easily assayed. Thus, we envision that each time one contemplates searching for an enzyme, the first step will be to construct an encoded reaction cassette to detect reactivity.

Finally, this technique may be important for physical organic chemists in that the rate of very slow reactions can be measured. Thus, one may learn about minor pathways that were thought to be highly disfavored or even forbidden.

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