Enzymatic amplification in a bioinspired, autonomous signal cascade†

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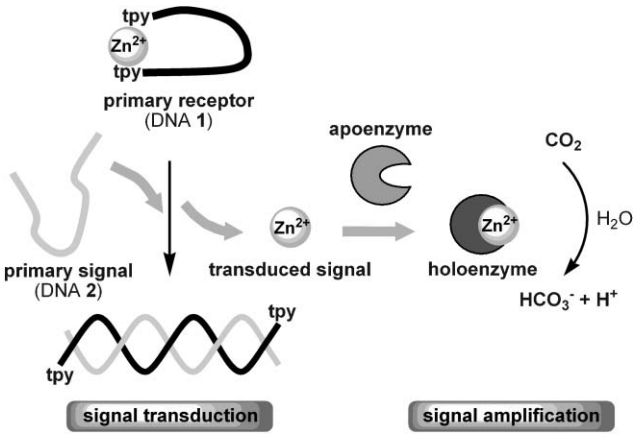
A two-step reaction cascade is applied to the sequence-specific detection of DNA by enzymatic amplification of the molecular into an optical signal.

Application of nature-inspired, allosteric signal transduction strategies to the development of novel bio- and chemosensors is an emerging research field.[1] Sensing in living systems depends, on the cellular level, on signal cascades, *i.e.* a chain of steps which usually results in a small stimulus eliciting a large response. Such cascades combine several signal transduction and amplification steps.

We have recently described the prototype of an autonomous‡ two-step artificial signal cascade which includes allosteric transduction and catalytic amplification.[2] The concept was applied to the sequence specific detection of DNA, the "primary signal", which triggers the release of a metal ion (Cu^{2+}), and the latter assembles as a cofactor with a precatalyst into the active, signal-amplifying chemical catalyst. A significant limitation is the low turnover frequency of the chemical catalyst which results in poor amplification and a long response time.

We are therefore exploring the re-activation of apoenzymes by their metal ion cofactors[3] as an alternative amplification strategy (Scheme 1), taking advantage of the modular conception such that transducing and catalytic unit can be independently optimized.[2] Carbonic anhydrase (CA), a zinc-containing enzyme, was selected due to its very high turnover rate, in the reversible hydration of CO_2 to HCO_3^-. In addition, CA catalyzes the hydrolysis of certain esters, although with much lower efficiency. The zinc free apoenzyme was prepared by dialysis in the presence of chelating ligands, following reported protocols.[4]

As described previously,[5] 20-mer DNA **1** modified by the tridentate chelator 2,2′:6′,2″-terpyridine (tpy) at both 3′ and 5′ terminus, forms a very stable circular 1 : 1-complex with Zn^{2+} ions, in which the metal ion is coordinated by both tpy moieties. On addition of complementary DNA **2**, the bis-chelation of the metal ion is disrupted due to a conformational change by formation of the rigid double helix and the metal complex is destabilized (negative allostery, Scheme 1). When apo-carbonic anhydrase is present in the reaction mixture in μM concentration, it binds the released Zn^{2+} since apo-CA has a much higher affinity to Zn^{2+} (log K = 10^{12})[6] than terpyridine (log K = 10^6).[7] Activity of the holoenzyme is monitored by hydrolysis of the fluorogenic ester



Scheme 1 The two-step reaction cascade includes allosteric signal transduction (DNA **2** to Zn^{2+}) and enzymatic signal amplification, with optical detection of the pH decrease by a color indicator. DNA **1** 5′-**tpy**d(ATCGTTACCAAAGCATCGTA)**tpy**, complementary DNA **2** 5′-d(TACGATGCTTTGGTAACGAT), mismatch DNA **3** 5′-d(TACGATGCTTTGGTAA***T***GAT).

substrate diacetyl fluorescein[8] (Fig. 1). Reactivation of CA within minutes is indicated by the increase of reaction rate with time, and is in accordance with a second order association constant k_2 = 10^4 M^{-1} s^{-1} of apo-CA and Zn.[9]

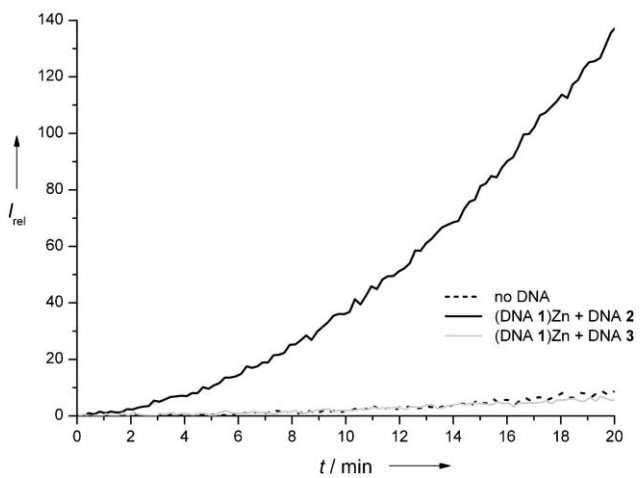


Fig. 1 Cleavage of diacetyl fluorescein, increase of relative fluorescence (λ_{ex} = 490 nm, λ_{em} = 514 nm) with time. Reaction solutions contain 1 μM apocarbonic anhydrase, 10 μM diacetyl fluorescein, 0.1 M NaCl, 10 mM $MgCl_2$ at pH 7.4 (0.1 M Trizma HCl) and 25 °C, 1 μM DNA (**1**)Zn, 2 μM complementary DNA **2** or mismatch DNA **3**, respectively. Reproducibility within ±20%.

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† Electronic supplementary information (ESI) available: Preparation of apoCA and instructions for the color assay with phenol red. See DOI: 10.1039/b611319b

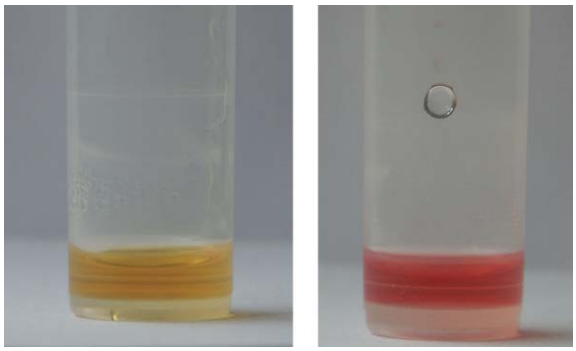

Fig. 2 CO_2-saturated solutions containing 0.1 M NaCl, 10 mM $MgCl_2$, apo-CA (0.25 μM), phenol red (17.5 μM) and (**1**)Zn (1 μM) at 0 °C. Reaction is initiated by addition of HEPES buffer (12.5 mM) and pictures are taken after 30 s. Left: In the presence of DNA **2** (1.1 μM), pH = 6.3. Right: in the absence of DNA **2**, pH = 8.5.

The presence of complementary DNA **2** is readily detected (Fig. 1) while single-mismatch DNA **3** has virtually no effect on ester cleavage rate. Residual activity of the apoenzyme sample contributes to a significant background reaction (Fig. 1) which limits the sensitivity of the assay to about 0.1 μM target DNA. Investigation of the apoenzyme sample by AAS revealed a Zn content of 0.05 equiv. Zn per enzyme and confirms that zinc extraction by standard dialysis protocols is incomplete.[3] In a control experiment, no reactivation of CA is observed when Zn^{2+} is replaced by Cd^{2+} in the experiment shown in Fig. 1. Affinity of Cd to apoCA[6] and tpy,[7] respectively, is similar to that of Zn but Cd does not restore esterase activity when added to apoCA at neutral pH.[10]

Next, we studied the conversion of the "natural" CA substrate CO_2. Hydration of CO_2 was indirectly monitored according to a literature protocol, observing the decrease of pH by the color change of the indicator phenol red (pK_a = 7.3, red at pH > 8.3, yellow at pH < 6.3).[11] Depending on the amount of active CA, CO_2 is converted more or less rapidly to HCO_3^- and H^+, resulting in protonation of a buffer (HEPES), decrease of pH and color change of the indicator from red to yellow (Fig. 2). In an assay containing 12.5 mM HEPES, apo-CA (0.25 μM), (**1**)Zn (1 μM) and complementary 20-mer DNA **2** (1.1 μM), the color change (initial pH 9.0 to final pH 6.3) takes 28 (±5) s, compared with 55 (±5) s in the presence of mismatched DNA **3** or 75 s in the absence of target DNA.

The amplification factor, *i.e.* the number of converted CO_2 molecules per target DNA **2** molecule, was estimated using pK_a = 7.6 for HEPES buffer. During a pH drop from 9.0 to 6.3, a minimum of 11 mM protons was released by the action of CA. Thus, conversion of 10 000 CO_2 molecules is triggered by one molecule of target DNA after 30 s in the experiment shown in Fig. 2, left. Again the background reaction, both by spontaneous conversion of CO_2 and by residual activity of apo-CA limits sensitivity of the assay to about 0.1 μM target DNA.

In conclusion, we have demonstrated that zinc cofactor dependent enzymes are effective catalytic modules of artificial signal cascades, yielding much higher amplification factors and shorter response times than chemical catalysts. High background reaction by both spontaneous substrate conversion and residual activity of the apoenzyme, limits the sensitivity of the CA based assay. We are currently trying to address this problem by using zinc enzymes which are active toward more robust substrates (*e.g.* peptidases), and by improving the protocols for apoenzyme generation.

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Notes and references

‡ In the present context, an autonomous signal cascade is considered a sequence of chemical reactions which—once initiated—proceeds without any intervention and yields a detectable physical and chemical signal.

1 A. Villaverde, *FEBS Lett.*, 2003, **554**, 169; R. R. Breaker, *Curr. Opin. Biotechnol.*, 2002, **13**, 31–39; L. Zhu and E. V. Anslyn, *Angew. Chem., Int. Ed.*, 2006, **45**, 1190.
2 N. Graf, M. Göritz and R. Krämer, *Angew. Chem., Int. Ed.*, 2006, **45**, 4013; In other autonomous enzyme-based assays for amplified DNA detection, target DNA binding directly interferes with enzyme–inhibitor or enzyme–cofactor interaction rather than triggering a reaction cascade: A. Saghatelian, K. M. Guckian, D. A. Thayer and M. R. Ghadiri, *J. Am. Chem. Soc.*, 2003, **125**, 344; P. Simon, C. Dueymes, M. Fontecave and J.-L. Décout, *Angew. Chem., Int. Ed.*, 2005, **44**, 2764; V. Pavlov, B. Shlyahovsky and I. Willner, *J. Am. Chem. Soc.*, 2005, **127**, 6522.
3 A. O. Udom and F. O. Brady, *Biochem. J.*, 1980, **187**, 329; Reactivation of zinc dependent apoenzymes has been applied to the detection of Zn^{2+}: N. Demir and Y. Demir, *Phytochem. Anal.*, 2004, **15**, 382; L. E. Grishina, N. A. Poskonnaya and T. N. Shekhovtsova, *Anal. Lett.*, 2000, **33**, 2197; K. Kobayashi, K. Fujiwara, H. Haraguchi and K. Fuwa, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 1932.
4 J. B. Hunt, M.-J. Rhee and C. B. Storm, *Anal. Biochem.*, 1977, **79**, 614.
5 M. Göritz and R. Krämer, *J. Am. Chem. Soc.*, 2005, **127**, 18016.
6 S. Lindskog and P. O. Nyman, *Biochim. Biophys. Acta*, 1964, **85**, 462.
7 R. H. Holyer, C. D. Hubbard, S. F. A. Kettle and R. G. Wilkins, *Inorg. Chem.*, 1966, **5**, 622.
8 J. D. Cohen and H. D. Husic, *Phytochem. Anal.*, 1991, **2**, 60.
9 R. W. Henkens and J. M. Sturtevant, *J. Am. Chem. Soc.*, 1968, **90**, 2669.
10 H. Strasdeit, *Angew. Chem., Int. Ed.*, 2001, **40**, 707.
11 W. Bruns and G. Gros, in *Carbonic Anhydrases*, ed. S. J. Dodgson, Plenum Press, New York, 1991, pp. 127–131.

