Chemical Amplification through Template-Directed Synthesis

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Template-directed polymerization reactions evolved as the biological strategy for information storage. Alternate coupling strategies[1] and RNA enzymes[2] exploit template-directed polymerization and/or oligomerization, but the ligation steps are limited byproduct release. Many different nucleic acid backbone linkages have been studied over the last several years in an attempt to develop metabolically stable antisense agents, and each of these linkages convey unique properties to the oligonucleotide duplex.[3] This insight into the role of the linkage has been critical to our design of a strategy which enables the amplification of the information encoded on a DNA template into a backbone-altered oligomer.

A ligation reaction that occurs through a reversible imine condensation of dCGT-CH_2CHO with H_2N-dTGC in the presence of a complementary DNA template is represented as a thermodynamic cycle in Figure 1. The equilibrium constants, K_{1-4}, originally estimated,[4a] are determined directly from ^{1}H and ^{15}N NMR experiments with a synthetic ^{15}N-enriched amine trimer.[5] With each substrate, amine and aldehyde trimers, at 7.5 mM in 500 mM NaCl at pH 6 and 25 °C, the imine-modified hexamer was below the limit of detection and $K_4 \leq 10^{-4}$ M^{-1}. On addition of 1 mol equiv of the DNA template, both the free amine and the imine were detected with ^{1}H−^{15}N HMQC experiments.[6] The carbinolamine intermediate was not detected under any conditions and is not shown in Figure 1.

The ^{1}H NMR chemical shift of the thymidine methyl groups which flank the reaction site were the most valuable reporters for the self-assembly process. Two sets of methyl signals were detected between 25 and 50 °C in slow exchange on the NMR time scale. The downfield set was assigned to the free trimers by comparison to spectra obtained in the absence of template.

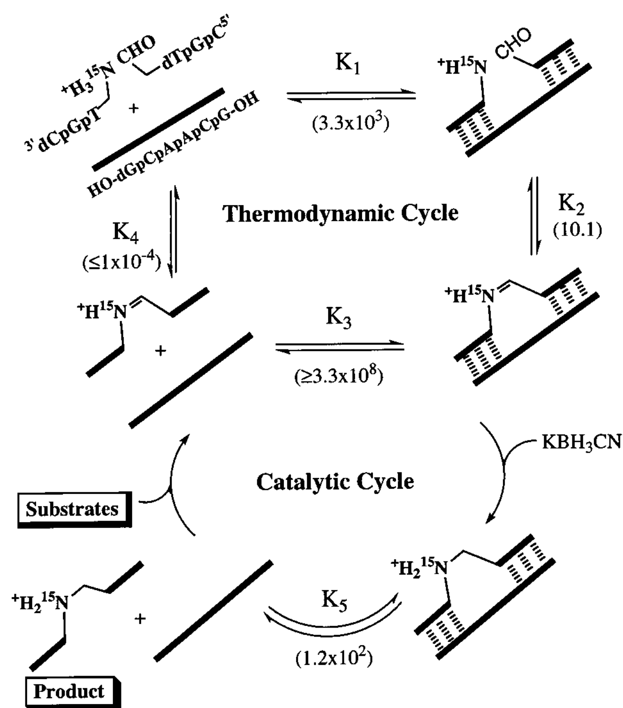

Figure 1. The thermodynamic and catalytic cycles for the DNA-catalyzed reductive amination with the determined equilibrium constants.

The chemical shifts of the high-field methyls were temperature dependent and assigned, based on comparisons with unmodified trimers, to the ternary and binary complexes, which are in rapid exchange via intramolecular condensation, K_2. A plot of the chemical shift of these lines as a function of temperature provides a lower limit on the melting curve for the imine duplex (Figure 2), while integration of the sets of methyl signals provides a direct measurement of K_1. The association equilibrium constant, K_1, is not appreciably different from that of two DNA trimers of the same sequence, and the condensation reaction, K_2, stabilizes the complex an additional 10-fold. K_3

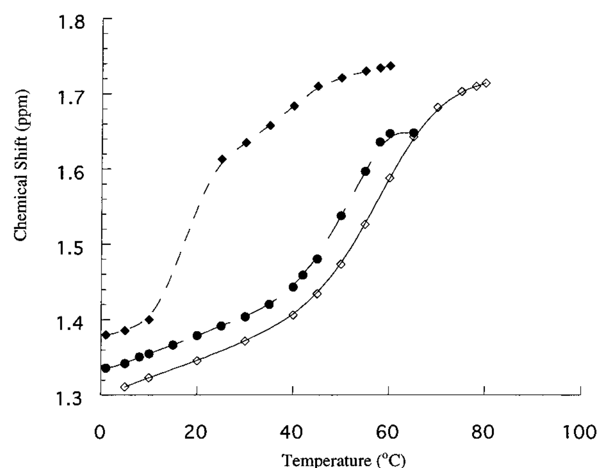

Figure 2. Comparison of the melting curves for the amine-modified (♦), native (◇), and imine-modified (●) duplexes determined from ^{1}H NMR chemical shift of the T-3 methyl signals. All NMR samples were eluted through Dowex 50WX2−100 (Na^+) ion-exchange resin, placed NaH_2PO_4, pH = 6, lyophilized 2−3 times from D_2O, and resuspended in D_2O to give a final concentration of 75 mM substrate in 10 mM buffer. The missing points in the amine-modified duplex result from exchange broadening at that temperature (10−25 °C) and inaccurate integrals. The extrapolated line is consistent with measurements at slightly lower concentrations.[4c]

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(5) The [^{15}N]thymidine was prepared via Mitsinobu coupling of [^{15}N]phthalimide (98 at. %) with 3′-TMS-thymidine and deprotected to obtain [5′-^{15}Naminothymidine.[4c] The [5′-^{15}N]amino-modified trimer ([5′-$^{15}N^+H_3$]-dTGC) was prepared via solid-phase synthesis: ^{1}H NMR (500 MHz, D_2O, pH 6) δ 8.01 (s, 1H, G-H8), 7.67 (d, J = 7.5 Hz, 1H, C-H6), 7.17 (s, 1 H, T-H6), 6.24 (dd, J = 6.5, 1H, G-H1′), 6.06 (dd, J = 6.5, 1H, C-H1′), 5.79 (dd, J = 6.5, 1H, TH1′), 5.73 (d, J = 7.5, 1H, C-H5), 4.93 (br s, 1H, G-H3′), 4.56 (br m, 1H, T-H3′), 4.34 (s, 1H, C-H3′), 4.25−4.00 (broad m, 7H, T-H4′, G-H4′, G-H5′, G-H5″, C-H4′, C-H5′, C-H5″), 3.40−3.00 (2m, 2H, T-H5′, 5″), 2.74 (2m, 2H, C-H2′, 2″), 2.30-2.19 (2m, 2H, T-H2′, 2″), 1.73 (s, 3H, T-CH_3). The coupling constant, $^3J_{(N, H)}$ of ^{15}N with T-H4′ at 1.8 Hz, was resolved on a Varian Unity 600; the chemical shift of T-H4′ was assigned at 4.23 ppm.

(6) The free amine nitrogen resonated at δ 25.1 and was spin−spin coupled to the 5′, 5″, and 4′ protons; the imine nitrogen resonated at δ 168.2 and was coupled to the imine proton at δ 8.03.

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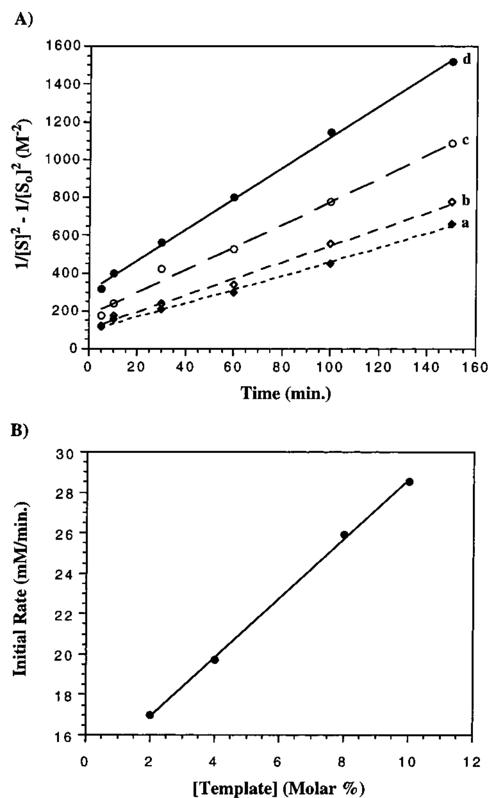

Figure 3. The substrates, 75 mM, were equilibrated with 0.01−0.1 mol equiv of the DNA template at pH 6 and 25 °C before 3 equiv of KBD_3CN was added to initiate the reaction. Reaction progress was monitored isocratically with a Zorbax Oligo reverse phase column eluted with 4:1 20 mM NaH_2PO_4/acetonitrile, pH 6.6; peak areas were determined with a Rainin Dynamax UV-D II detector and software. (A) Amine formation with time fit to a third-order rate expression with the molar percent of template at (♦) 1%, (◊) 2%, (○) 4%, and (●) 8%. (B) Plot of the initial rate of amine formation as a function of template concentration.

was calculated from the thermodynamic cycle using the limiting value of K_4 and the measured values of K_1 and K_2.

In a reducing environment,[7] the equilibrating imine is trapped as the amine hexamer, Figure 1. The reaction shows third-order kinetics, Figure 3A; with excess reductant, it is first order in both substrates (data not shown) and template, Figure 3B. With only 0.01 mol equiv of DNA, which corresponds to 91% template saturation under these conditions, the turnover number is 13 min^{-1}. There is no product inhibition, at least through the first half-life where the product-to-template ratio is >50:1. The product duplex, K_5, destabilized due to the increased flexibility in the alkylamine linkage,[4c] is ≥ 10^6-fold weaker than the substrate duplex (Figure 1), and product inhibition should never be significant. Engineered ribozymes are limited by slow product release.[8a,b] In these cases, the introduction of mismatches[8c] or tertiary interactions[8d] have been used to increase turnover rates, but such changes lower fidelity and require much larger MW catalysts. Both of the exchange rates, estimated from line broadening analyses of the NMR spectra from Figure 2, and the effects of different reductants,[7] establish the chemical step as rate limiting, and its improvement will greatly enhance both fidelity and overall turnover. It is therefore now possible to envision the storage and amplification of information encoded on a DNA template by translation[9] into backbone-modified polymers

It has been suggested that an array of oligomeric species capable of catalyzing many different reactions evolved prior to the development of self-sustainability.[10] Polymerization reactions require a vast excess of monomers, and for a prebiotic template to be efficiently self-sustainable, the reflexive catalyst must be capable of creating a “metabolism” sufficient for monomer generation. Here, we provide a pathway for a short oligomer to function as a catalyst to translate its encoded information into a different oligomer, information that is amplified manyfold without product inhibition. Imine coupling/reduction is certainly not the only reaction that can ligate oligomers and probably just the first of many backbone modifications that can be catalytically ligated by a template. Both DNA and RNA catalyze chemical reactions,[11] and the 2′-OH of RNA enriches the chemical reactivity. The placement of a basic amine in the backbone further extends the functional groups present in the nucleic acids, and the catalytic activity can be expected to be more diverse. These backbone ligations therefore provide a mechanism for a single oligomer to nucleate the growth in structural diversity of catalytic templates.

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(7) Several different mild reducing conditions were investigated including cyanoborohydrides Bu_4NBH_3CN, KBD_3CN, and $NaBH_3CN$ and the borohydride-base complexes BH_3-2,6-lutidine and BH_3−pyridine. Each proved to be chemoselective but gave different overall rates.

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