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Factors influencing hairpin oligonucleotide cyclization by the uncatalyzed alkyne-azide cycloaddition (AAC) reaction*

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Abstract: Cyclization of short hairpin oligonucleotides (ODNs) containing unactivated alkynes and alkyl azides produces triazole-linked products slowly in the absence of Cu^I catalysis. The factors affecting the rate of this cycloaddition reaction have been investigated on a variety of hairpin sequences. The reaction proceeds best at intermediate concentrations of sodium chloride, but is inhibited by phosphate and Tris buffers. It is accelerated by increasing the temperature provided that Watson–Crick base pairing can occur. The rationale for these observations is discussed.

Keywords: AAC reaction; alkyne azide; DNA cyclization; hairpin; uncatalyzed.

INTRODUCTION

Click chemistry was developed to provide a simple method to join together organic molecules in high yield under mild conditions in the presence of a diverse range of functional groups [1]. The best example of this new class of extremely efficient reactions is the Cu^I-catalyzed (3 + 2) azide-alkyne cycloaddition (CuAAC) reaction [2,3]. We have a long-standing interest in using new synthetic methods to construct chemically modified DNA oligonucleotides (ODNs) for biological and nanotechnology applications, and it was clear to us that the CuAAC reaction has great potential in this context. The click reaction is useful in DNA strand ligation and other biological applications because azides and unactivated alkynes are almost entirely unreactive toward the functional groups normally encountered in nature, therefore they can be attached to nucleic acids without greatly disturbing their biophysical properties. In addition, the triazole unit is extremely stable and nontoxic. In the past three years, the CuAAC reaction has been used extensively to label ODNs with a variety of groups including fluorescent dyes [4–8], sugars [4,9], and peptides [10]; to construct cyclic single- and double-stranded DNA [11,12]; and to produce analogs of DNA with modified nucleobases [5,6,13–15] and backbones [16–19]. It has been rapidly adopted in the nucleic acids field due to its remarkably high efficiency. The requirement for Cu^I imposes limitations on potential applications. Indeed, there are cases where the use of Cu^I is not possible, particularly for in vivo studies, as copper is toxic. The uncatalyzed (AAC) reaction has therefore been studied as an alternative for biological applications. It can be greatly accelerated by the use of activated alkynes with attached electron-withdrawing groups, a principle which has been demonstrated in cell surface labeling with carbohydrates [20]. This approach is feasible in cases where a large excess of

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the alkyne component is used, but is not practical when a stoichiometric ratio of alkyne and azide is required to react. This is because activated alkynes undergo reactions with nucleophiles and therefore have a limited lifetime in biological media. We are interested in developing chemical methods of ligating nucleic acid strands in vivo, and for this process a 1:1 ratio of the two reacting species is required. As the use of copper catalysis and activated alkynes is precluded, we decided to investigate the DNA-templated AAC reaction on unactivated (stable) alkynes. The aim of this study is to determine the effects of the properties of the interacting ODNs and reaction conditions on reaction rate. In the work presented here we have varied the salt concentration, the nature of the buffer, and temperature to reveal some interesting trends. These model studies focus on the cyclization of short hairpin-loop ODNs in which the stoichiometry between alkyne and azide, and the templating factor (Watson–Crick base pairing) are built into the system.

RESULTS AND DISCUSSION

We have previously shown that the CuAAC reaction is an efficient method to close hairpin loops [12,21], ligate adjacent strands bound to a common template [11], and cross-link sequences annealed to each other [22]. The importance of this work increased greatly when we recently demonstrated that triazole linkages in DNA strands can be read through during polymerase chain reaction (PCR) [19]. Thus, a variety of interesting biological applications become feasible, including the chemical synthesis of very long DNA strands via click DNA ligation, and in vivo templated ODN ligation for antisense or RNAi-based gene inhibition. With such biological applications in mind, we are investigating the AAC reaction in the absence of Cu^{I}. Our initial studies focused on the hairpin-loop sequence K-CGC-X-X-GCG-Z, where K = propargyl alkyne **1a**, Z = azide **1c**, and X = hexaethylene glycol monomer (Fig. 1).

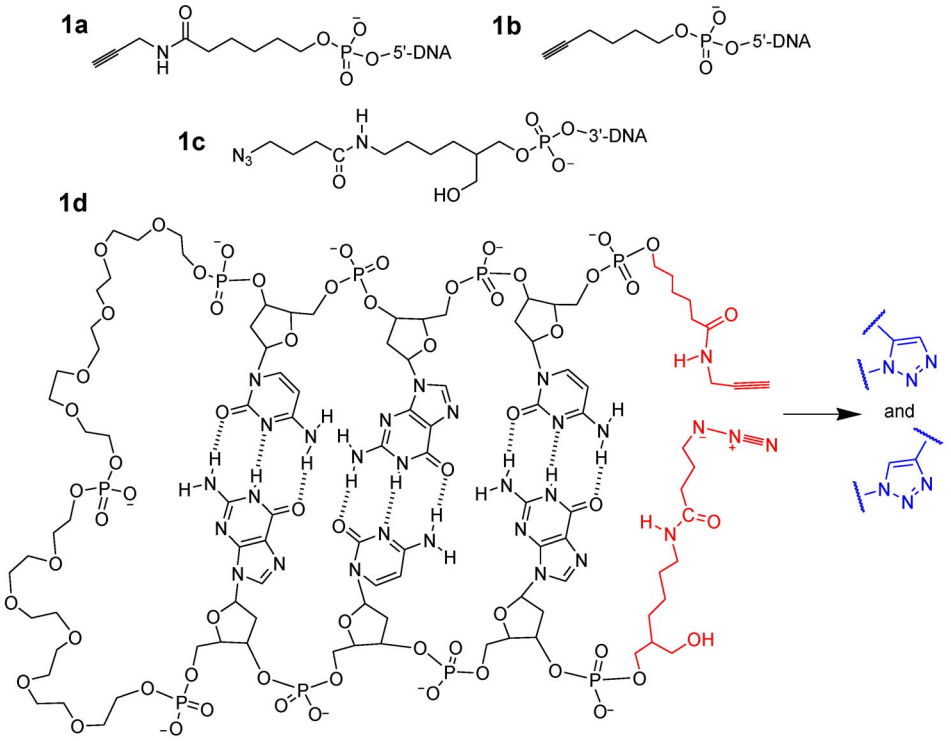

Fig. 1 The DNA-templated AAC reaction to cyclize the hairpin-loop sequences such as K-CGC-X-X-GCG-Z (above) in which K = propargyl alkyne **1a**, Z = azide **1c**, and X = hexaethylene glycol.

The *bis*-hexaethylene glycol linker provides a simple means of constructing a thermodynamically stable hairpin loop. In this system, the alkyne and azide are held in close proximity by the three GC base pairs, provided that the conditions for the reaction are compatible with duplex formation. The alkyne of propargylamide is stable in aqueous buffer (i.e., it does not react rapidly with electrophiles) and can therefore be considered to be an "unactivated" alkyne. The melting temperature of this mini duplex in 0.2 M sodium chloride is 58 °C, and a proportion of the individual molecules exists in the double-stranded form up to 80 °C (Fig. 2c).

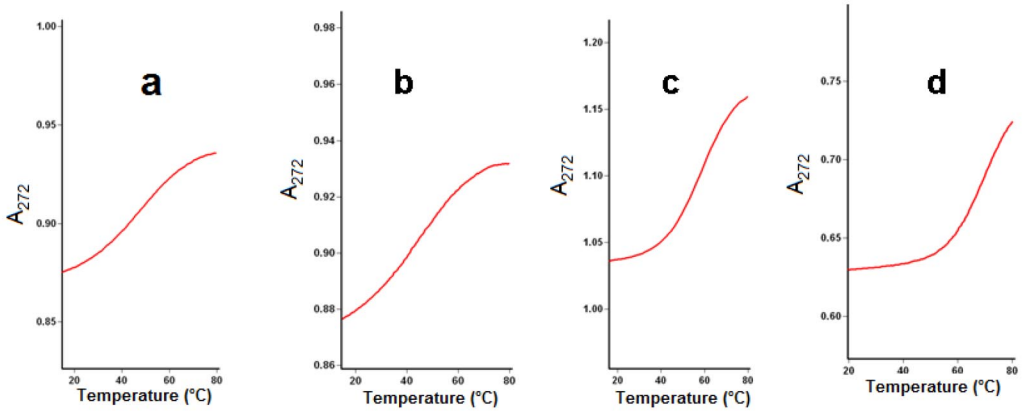

Fig. 2 UV melting experiments on hairpin ODNs used in this study. (a) K-GC-X-GC-Z (K = propargyl alkyne **1a**, T_m = 49 °C); (b) K-GC-X-GC-Z (K = hexynol **1b**, T_m = 45 °C); (c) K-CGC-X-X-GCG-Z (K = propargyl alkyne **1a**, T_m = 58 °C); and (d) K-CGGC-XX-GCCG-Z (K = propargyl alkyne **1a**, T_m = 71 °C). In all cases, X = hexaethylene glycol monomer. UV melting curves were measured on Cary 400 Scan UV-Visible Spectrophotometer (Varian) at 7–10 μM concentration of ODN in 10 mM sodium phosphate buffer, 200 mM NaCl, and pH 7.0. Spectra were recorded at 272 nm. T_m values were calculated using Cary Win UV thermal application software.

The AAC reaction to cyclize this hairpin was studied in the presence and absence of sodium phosphate buffer (pH 7.0) and sodium chloride over a range of temperatures. The reaction proved to be very sensitive to salt and temperature. The presence of sodium chloride and a temperature of at least 37 °C were required to achieve an observable reaction (Fig. 3A), but phosphate buffer was inhibitory and no significant reaction was observed at room temperature. A mixture of 1,4 and 1,5 triazoles would be expected in the absence of copper but these isomers were not resolved on the polyacrylamide gels. A related study was carried out on a shorter hairpin sequence K-GC-X-GC-Z (K = propargyl alkyne **1a**) with a single hexaethylene glycol loop (Fig. 4). The melting temperature of this hairpin is 49 °C (Fig. 2a), and in this case the reaction temperature range was extended to 80 °C. The reaction proceeded best at the highest temperature and at 200 mM sodium chloride. It was not successful in water, and was very slow in 1 M sodium chloride. This was confirmed for the three base-pair duplexes by gel and capillary electrophoresis (Figs. 3B,C). The negative result in water is not unexpected, as DNA base pairing does not occur in the absence of salt, so the templating factor is absent. The lack of reactivity at high salt is less easy to rationalize, as the melting temperature of DNA duplexes is known to increase as a function of salt concentration. It is possible that high ionic strength leads to a rigid duplex which lacks the necessary flexibility to allow the alkyne and azide to come into close proximity. Alternatively, the hydrophobic linkers on which the alkyne and azide are appended might collapse in high salt conditions and cause these reactive groups to become inaccessible. In an attempt to understand the effect of sodium chloride concentration on reaction rate, hairpin duplex stability was determined in unbuffered NaCl at 0.2 and 1.0 M (i.e., optimum and sub-optimum AAC reaction conditions). The T_m increased by less than

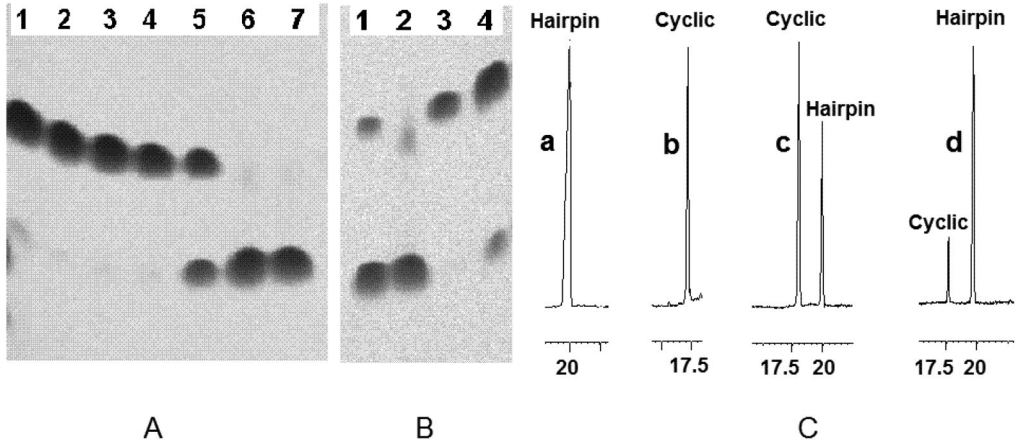

Fig. 3 Attempts to cyclize the hairpin-loop sequence K-CGC-X-X-GCG-Z. (K = propargyl alkyne). **3A** and **3B**, denaturing 20 % PAGE gel, higher-spot-uncyclized hairpin, lower-spot-cyclized product. **3C** capillary electrophoresis (CE). ODN concentration = 10 μM. **3A:** lane 1: buffer, 50 °C, 1 day; lane 2: buffer, RT, 1 day; lane 3: buffer, 37 °C, 1 day; lane 4: 0.2 M NaCl, RT, 1 day; lane 5: 0.2 M NaCl, 37 °C, 1 day; lane 6: buffer, RT, with Cu^I, 2 h; lane 7: 0.2 M NaCl, using Cu^I, RT, 2 h. Buffer = 10 mM sodium phosphate with 0.2 M NaCl at pH 7. **3B:** lane 1: 0.2 M NaCl, 80 °C, 1 day; lane 2: control reaction using Cu^I , RT, 2 h; lane 3: control starting oligo; lane 4: 1.0 M NaCl, 80 °C, 1 day. **3C:** CE for cyclization reactions. (a) Control starting oligo. (b) Reaction mixture using Cu^I, RT, 2 h. (c) Reaction mixture without Cu^I, 0.2 M NaCl, 80 °C, 1 day. (d) Reaction mixture without Cu^I, 1 M NaCl, 80 °C, 1 day. ssDNA 100-R Gel, Tris-Borate-7 M Urea on a Beckman Coulter P/ACE^{TM} MDQ Capillary Electrophoresis System using 32 Karat software. Injection voltage 10.0 kv and separation voltage 9.0 kv. *X*-axis is time (min), *Y*-axis is UV absorbance at 254 nm.

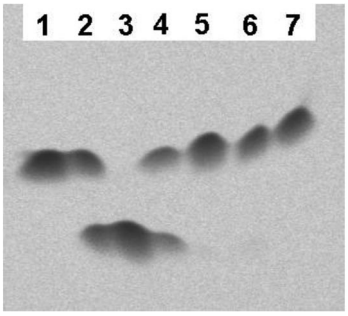

Fig. 4 Attempts to cyclize the hairpin-loop sequence K-GC-X-GC-Z (K = propargyl alkyne **1a**). Denaturing 20 % PAGE gel. Higher-spot-uncyclized hairpin, lower-spot-cyclized product. Lane 1: control starting oligo; lane 2: 1 M NaCl, 80 °C, 2 days; lane 3: 0.2 M NaCl, 80 °C, 2 days; lane 4: 0.2 M NaCl, 37 °C, 1 day; lane 5: 0.2 M NaCl, RT, 1 day; lane 6: water, 80 °C, 1 day; lane 7: water, 37 °C, 1 day. ODN concentration = 5 μM (0.2 OD_{260} units in 1 ml reaction mixture).

2 °C over this range (data not shown), a much smaller increase than calculated for a normal GC-rich DNA duplex (~10 °C). Although this result is counter-intuitive, it is consistent with the observation that the activation energy for DNA hairpin closure increases as a function of NaCl concentration [23].

Although 80 °C is above the melting temperature of the duplex, it is not too high to prevent transient duplex formation. The reaction needs heat for activation, but it also requires templating to hold the alkyne and azide in close proximity. To test this, we repeated the above experiment over a wider temperature range under the optimum salt conditions of 0.2 M NaCl (Fig. 5). The best results were obtained

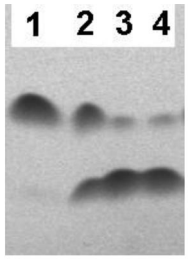

Fig. 5 Attempts to cyclize the hairpin-loop sequence K-GC-X-GC-Z (K = propargyl alkyne **1a**) at different temperatures. Denaturing 20 % PAGE gel. Higher-spot-uncyclized hairpin, lower-spot-cyclized product. Lane 1: control starting oligo; lane 2: 40 °C, 1 day; lane 3: 60 °C, 1 day; lane 4: 80 °C, 1 day. ODN concentration = 5 μM (0.2 OD_{260} units in 1 ml reaction mixture).

between 60 and 80 °C. In contrast, little reaction was observed at 40 °C. This supports the hypothesis that both heat and templating are necessary for the reaction. We cannot discount the possibility discussed above that flexibility of the DNA backbone is a factor, in which case the reaction will be faster around the T_m of the duplex rather than at lower temperatures when the duplex is more rigid.

An experiment was conducted to determine the optimum salt concentration for the reaction (Fig. 6). Good results were obtained between 0.15 and 0.4 M NaCl, but lower and higher salt concentrations gave poor reactivity. A series of experiments with the longer K-CGGC-XX-GCCG-Z duplex revealed similar trends (Fig. 7). The reaction was inhibited by Tris and phosphate buffers, but required sodium chloride, and proceeded much better at 0.2 M NaCl than at 1.0 M NaCl. It is important to note that the sodium chloride used in these experiments did not contain Cu^I. Moreover, traces of other metal ions cannot explain the apparent requirement for NaCl because the reaction was slow at high NaCl concentrations, and even in the presence of NaCl the reaction was inhibited by phosphate and Tris buffers.

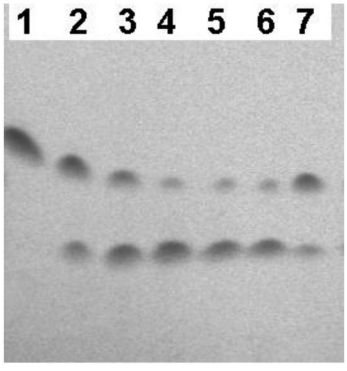

Fig. 6 Attempts to cyclize the hairpin-loop sequence K-GC-X-GC-Z (K = propargyl alkyne **1a**) at different concentrations of NaCl. Denaturing 20 % PAGE gel. 80 °C 1 day. Higher-spot-uncyclized hairpin, lower-spot-cyclized product. Lane 1: control starting oligo; lane 2: 50 mM NaCl; lane 3: 100 mM NaCl; lane 4: 150 mM NaCl; lane 5: 200 mM NaCl; lane 6: 400 mM NaCl; lane 7: 600 mM NaCl. ODN concentration = 3 μM.

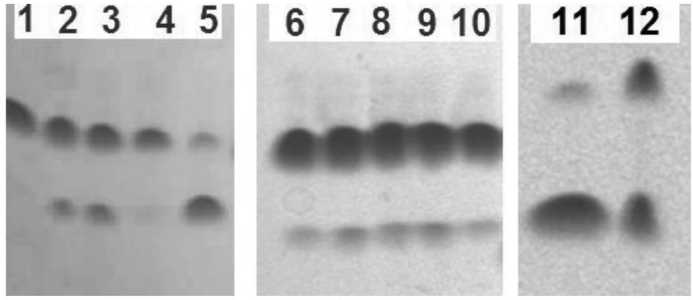

Fig. 7 Attempts to cyclize the hairpin-loop sequence K-CGGC-XX-GCCG-Z (K = propargyl alkyne **1a**) under various conditions. Denaturing 20 % PAGE gel. All reactions carried out at 80 °C for 2 days. Higher-spot-uncyclized hairpin, lower-spot-cyclized product. Lane 1: control starting oligo; lane 2: Tris buffer, 0.2 M NaCl, pH 7; lane 3: sodium phosphate buffer, 0.2 M NaCl, pH 7; lane 4: water; lane 5: 0.2 M NaCl; lanes 6–10 sodium phosphate buffer. Lane 6: pH 8, 0.2 M NaCl; lane 7: pH 8, 1.0 M NaCl; lane 8: pH 7, 0.2 M NaCl; lane 9: pH 7, 1.0 M NaCl; lane 10: pH 6.2, 0.2 M NaCl; lanes 11 and 12: no buffer. Lane 11: 0.2 M NaCl; lane 12: 1.0 M NaCl.

Finally, no significant reaction occurs for DNA sequences that cannot engage in duplex formation via base pairing. In contrast to the duplex-forming sequence K-GC-X-GC-Z, the corresponding "single base-pair" dinucleotide K-G-X-C-Z does not engage in detectable Watson–Crick hydrogen bonding, even at room temperature [12]. It failed to form a triazole after one day, and after two days only a small amount of cyclic product was observed. The duplex-forming properties of this sequence cannot be examined by ultraviolet melting because of the lack of base stacking, but NMR studies in H_2O show that it does not participate in inter-base hydrogen bonding [12], so no reaction templating is possible. Further support for the importance of duplex formation was obtained from attempts to cyclize 12-mer and 22-mer single-stranded ODNs which contain no base-paired regions. In the absence of Cu^I, the reaction was unsuccessful, even at 80 °C over two days (data not shown). However, as expected, it went to completion on the same ODNs in 2 h at room temperature in presence of Cu^I. The necessity for Watson–Crick base pairing was confirmed by comparing the rate of the reaction between duplex-forming hairpin K-GC-X-GC-Z (K = propargylamide **1a** or hexynol **1b**) and a similar ODN which cannot form base pairs, K-GC-X-CG-Z (K = hexynol **1b**). It is clear from the gel analysis (Fig. 8, lane 6) that the reaction is very slow for the ODN that cannot form a duplex. The same conclusion was obtained when the reaction was studied over a longer period of time (Fig. 9).

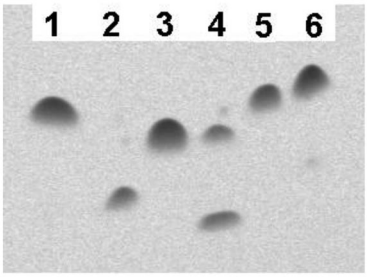

Fig. 8 Attempts to cyclize hairpin loops at 80 °C in 0.2 M NaCl. Denaturing 20 % PAGE gel. Higher-spot-uncyclized hairpin, lower-spot-cyclized product. Lane 1: control starting oligo K-GC-X-GC-Z (K = propagyl alkyne **1a**); lane 2: reaction mixture after 1 day, oligo K-GC-X-GC-Z (K = propagyl alkyne **1a**); lane 3: control starting oligo K-GC-X-GC-Z (K = hexynol **1b**); lane 4: reaction mixture after 1 day for oligo K-GC-X-GC-Z (K = hexynol **1b**); lane 5: control starting oligo K-GC-X-CG-Z (K = hexynol **1b** and no base pairing); lane 6: reaction mixture after 1 day for oligo K-GC-X-CG-Z (K = hexynol **1b** and no base pairing).

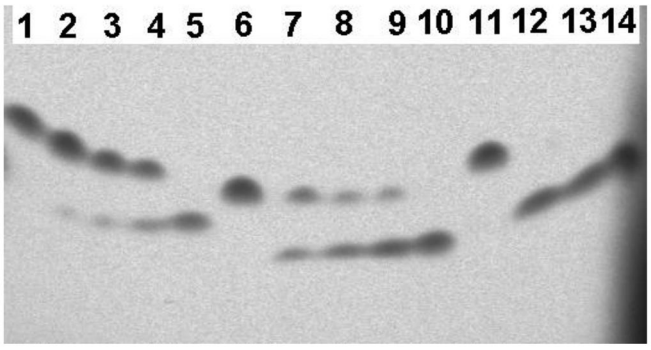

Fig. 9 Attempts to cyclize hairpin-loop ODNs in 0.2 M NaCl. Denaturing 20 % PAGE gel. All reactions without Cu^I at 80 °C (various timescales) and with Cu^I at RT for 2 h. Higher-spot-uncyclized hairpin, lower-spot-cyclized product. Lanes 1–5: K-GC-X-CG-Z (K = hexynol **1b** and no base pairing); lane 1: control starting oligo; lanes 2–4: reaction mixtures after 1, 2, and 3 days; lane 5: control reaction using Cu^I. Lanes 6–10 (K-CG-X-CG-Z, K = hexynol **1b**); lane 6: control starting oligo; lanes 7–9: reaction mixtures after 1, 2, and 3 days; lane 10: control reaction using Cu^I. Lanes 11–14 (K-CG-X-CG-Z, K = propargyl alkyne **1a**); lane 11: control starting oligo; lanes 12, 13: reaction mixtures after 1 and 2 days; lane 14: control reaction using Cu^I.

The relative reactivity of two alkynes was also demonstrated by comparing the cyclization of two similar ODNs with alkynes **1a** and **1b**. The rate of the reaction was faster when the more active propargyl alkyne (**1a**) was incorporated on the ODN compared with the less active one (**1b**), which lacks the adjacent mildly activating amide bond (Figs. 8, 9).

CONCLUSIONS

Our results show that uncatalyzed AAC reaction occurs on DNA base-paired templates in an optimal window of ionic strength (0.15–0.40 M NaCl). The rate of reaction increases as a function of temperature provided that enough time is spent in the base-paired state at temperatures moderately above T_m. The dependence on salt concentration suggests that dynamics have a profound influence on the templated AAC process, since the duplex flexibility is altered at 1 M NaCl relative to lower ionic strength. This could result in an increase in activation energy for hairpin closure at high salt [23]. An alternative explanation is inaccessibility of the alkyne and azide moieties in the presence of high concentrations of NaCl due to the hydrophobic effect. No significant reaction occurs for control DNA sequences that cannot engage in duplex base pairing, so it is clear that the DNA duplex is crucial in bringing the alkyne and azide into close proximity. It will be interesting to investigate the effects of divalent cations and duplex-stabilizing ligands (intercalators, groove binders) that might allow the templated reaction to be carried out at higher temperatures, and to carry out the AAC reaction in a more stable caged DNA environment. High-resolution NMR and molecular dynamics studies could shed more light on the importance of DNA duplex conformation and flexibility on the rate of the cyclization reaction.

EXPERIMENTAL

ODN synthesis and purification

Standard DNA phosphoramidites, solid supports, and additional reagents including C7-aminoalkyl CPG and DMT hexaethylene glycol phosphoramidite monomer were purchased from Link Technologies (Glasgow, Scotland) or Applied Biosystems (UK) Ltd. All ODNs were synthesized on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 1.0 μmol phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling effi-

ciencies and overall yields were determined by the automated trityl cation conductivity monitoring facility on the DNA synthesizer and in all cases were >98.0 %. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling times were 25 seconds for normal A, G, C, and T monomers and 10 min for the alkyne and hexaethylene glycol phosphoramidites. Cleavage of ODNs from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 °C. The ODNs were gel-filtered using disposable NAP-10 columns (GE Healthcare) according to the manufacturer's instructions then freeze-dried before labeling with the butyryl azide active ester. To label ODNs with the azide function, the NHS ester of 4-azidobutyric acid [19] (2 mg) was added to the 3'-amino-modified ODNs (1 μmol, freeze-dried) in 120 μl of DMSO:0.5 M Na_2CO_3/$NaHCO_3$ buffer 1:2 at pH 8.75, and the reaction was left to proceed for 4 h at room temperature. The fully labeled ODNs were gel-filtered using disposable NAP-10 columns, then purified by reversed-phase high-performance liquid chromatography (HPLC) (C8) in a gradient of acetonitrile in 0.1 M ammonium acetate and desalted by NAP-10 gel-filtration [12]. HPLC showed one peak indicating that the yield of the labeling reactions is >90 %.

General method for cyclization of ODNs in the absence of Cu^I

A solution of ODN (2–10 nmol) in 0.2 M NaCl (1 ml) was left at the desired temperature for a specific period of time. The solution was desalted using a Nap-10 gel-filtration column then freeze-dried before loading onto a 20 % polyacrylamide/7 M urea gel and electrophoresed at a constant power of 20 W for 3 h, using 0.09 M Tris-borate-EDTA buffer (pH 8.0). Matrix-assisted laser desorption/ionization with time-of-flight (MALDI-TOF) mass spectrometry confirmed that the cyclic constructs had the correct molecular weight. Capillary and gel electrophoresis showed that the uncatalyzed and Cu^I-catalyzed reactions gave the same products.

Cyclization of ODNs in the presence of Cu^I

For control cyclization reactions catalyzed by Cu^I, the above conditions were used with Cu^I generated in situ from sodium ascorbate and cupric sulfate [12]. The reaction mixture was left for 2 h at room temperature, desalted using a NAP-10 column, then freeze-dried before loading on the gel. MALDI-TOF and electrospray mass spectrometry confirmed that the hairpin and cyclic constructs had the correct molecular weights [12].

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