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Covalent Schiff Base Catalysis and Turnover by a DNAzyme: A M^{2+}-Independent AP-Endonuclease Mimic

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Abstract: A DNAzyme, synthetically modified with both primary amines and imidazoles, is found to act as a M^{2+}-independent AP lyase-endonuclease. In the course of the cleavage reaction, this DNAzyme forms a covalent Schiff base intermediate with an abasic site on a complementary oligodeoxyribonucleotide. This intermediate, which is inferred from $NaCNBH_3$ trapping as well as cyanide inhibition, does not evidently accumulate because the second step, dehydrophosphorylative elimination, is fast compared to Schiff base formation. The 5′-product that remains linked to the catalyst hydrolyzes slowly to regenerate free catalyst. The use of duly modified DNAzymes to perform Schiff base catalysis demonstrates the value of modified nucleotides for enhancing the catalytic repertoire of nucleic acids. This work suggests that DNAzymes will be capable of catalyzing aldol condensation reactions.

Introduction and Context

Covalent Schiff base formation between a proteinaceous alkyl amine (N-terminal, ϵ-Lys) and a substrate carbonyl represents a cornerstone in the catalytic repertoire of several enzyme classes that make and break carbon−carbon and carbon−heteroatom bonds. Quite notably, Schiff base catalysis is the *modus operandi* of the class I aldolases,[1] acetoacetate decarboxylases,[2] aldolase/ketosteroid isomerase antibodies,[3] and apurinic/apyrimidinic lyase-endonucleases (APEs).[4] In the case of APEs, an amine on the APE and the aldehyde of an abasic site on DNA dehydrate to form a Schiff base linkage that accompanies general base-catalyzed elimination of the 3′-phosphodiester linkage. The resulting 5′-product is then hydrolyzed to regenerate active enzyme.[5] A hallmark of Schiff base catalysis has been the irreversible reduction of the Schiff base by borohydride that results in a stable amino adduct.

Following Lindahl's early work on amine-promoted cleavage of apurinic DNA,[6] Helene and Laval independently identified the simple tripeptide Lys-Trp-Lys as the first APE mimic: it intercalates at an abasic site, forms a Schiff base, and catalyzes elimination resulting in strand scission.[7] This pioneering work suggested a "biomimetic" approach to generating synthetic molecules that would recognize DNA as a substrate and catalyze reactions typical of much larger protein enzymes. Since this discovery, recognition and cleavage at abasic sites by proteins, peptides, and dialkylamine-linked intercalators has been an important avenue of investigation.[8,9] To date, Schiff base-promoted lyase activity seems to be reserved for peptides, proteins, and various intercalators presenting nitrogen nucleophiles.

It is thus somewhat surprising that there has been no report of an alkylamine-oligonucleotide conjugate that promotes strand scission at an abasic site on a complementary sequence. Despite many reports of amine-modified oligonucleotides that address several contemporary interests,[10] to the best of our knowledge, Manoharan et al. describe the only relevant report of such an interaction, namely, the condensation of a 2′*O*-pentylamine-modified oligonucleotide with an abasic site of a complementary strand to form a Schiff base. Although trappable with $NaBH_4$, this construct remains idle with respect to elimination and

(1) Grazi, E.; Rowley, P. T.; Cheng, T.; Tchola, O.; Horecker, B. L. *Biochem. Biophys. Res. Commun.* **1962**, *9*, 38−43.
(2) Hamilton, G. A.; Westheimer, F. H. *J. Am. Chem. Soc.* **1959**, *81*, 6332−3.
(3) Wagner, J.; Lerner, R. A.; Barbas, C. F. *Science* **1995**, *270*, 1797−800.
(4) (a) Sun, B.; Latham, K. A.; Dodson, M. L.; Lloyd, R. S. *J. Biol. Chem.* **1995**, *270*, 19501−8. (b) Dodson, M. L.; Michaels, M. L.; Lloyd, R. S. *J. Biol. Chem.* **1994**, *269*, 32709−12. (c) Nash, H. M.; Lu, R. Z.; Lane, W. S.; Verdine, G. L. *Chem. Biol.* **1997**, *4*, 693−702. (d) Sidorkina, O. M.; Laval, J. *Nucl. Acids Res.* **1998**, *26*, 5351−7.
(5) (a) Lloyd, R. S.; Hanawalt, P. C.; Dodson, M. L. *Nucl. Acids Res.* **1980**, *8*, 5113−27. (b) Ganesan, A. K.; Seawell, P. C.; Lewis, R. J.; Hanawalt, P. C. *Biochemistry* **1986**, *25*, 5751−5. (c) Gruskin, E. A.; Lloyd, R. S. *J. Biol. Chem.* **1986**, *261*, 9607−13.
(6) Lindahl, T.; Andersson, A. *Biochemistry* **1972**, *11*, 3618−23.
(7) (a) Behmoaras, T.; Toulme, J. J.; Helene, C. *Nature* **1981**, *292*, 858−9. (b) Pierre, J.; Laval, J. *J. Biol. Chem.* **1981**, *256*, 10217−20.
(8) (a) Fkyerat, A.; Demeunynck, M.; Constant, J.-F.; Michon, P.; Lhomme, J. *J. Am. Chem. Soc.* **1993**, *115*, 9952−9. (b) References reviewed in: Lhomme, J.; Constant, J.-F.; Demeunynck, M. *Biopolymers* **1999**, *52*, 65−83.
(9) (a) Manoharan, M.; Mazumder, A.; Ransom, S. C.; Gerlt, J. A.; Bolton, P. H. *J. Am. Chem. Soc.* **1988**, *110*, 2690−1. (b) Mazumder, A.; Gerlt, J. A.; Rabow, L.; Absalon, M. J.; Stubbe, J.; Bolton, P. H. *J. Am. Chem. Soc.* **1989**, *111*, 8029−30. (c) Mazumder, A.; Gerlt, J. A.; Absalon, M. J.; Stubbe, J.; Cunningham, R. P.; Withka, J.; Bolton, P. H. *Biochemistry* **1991**, *30*, 1119−26.
(10) (a) Takeda, T.; Ikeda, K.; Mizuno, Y.; Ueda, T. *Chem. Pharm. Bull.* **1987**, *35*, 3558−67. (b) Prakash, T. P.; Barawkar, D. A.; Kumar, V.; Ganesh, K. N. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1733−8. (c) Schmid, N.; Behr, J. P. *Tetrahedron Lett.* **1995**, *36*, 1447−50. (d) Sund, C.; Puri, N.; Chattopadhyaya, J. *Tetrahedron* **1996**, *52*, 12275−90. (e) Nara, H.; Ono, A.; Matsuda, A. *Bioconj. Chem.* **1995**, *6*, 54−61. (f) Haginoya, N.; Ono, A.; Nomura, Y.; Ueno, Y.; Matsuda, A. *Bioconj. Chem.* **1997**, *8*, 271−80. (g) Shinozuka, K.; Matsukura, M.; Okamoto, T.; Sawai, H. *Nucleosides Nucleotides* **1998**, *17*, 2081−4. (h) Markiewicz, W. T.; Godzina, P.; Markiewicz, M.; Astriab, A. *Nucleosides Nucleotides* **1998**, *17*, 1871−80. (i) Potier, P.; Adib, A.; Kochkine, A.; Huc, I.; Behr, J. P. *Nucleosides Nucleotides* **1999**, *18*, 1467−8.

consequent strand cleavage.[11] This result suggested while a primary amine might be required for Schiff base formation, it is insufficient for catalyzing elimination and strand scission. This might be explained in contextual terms: the construct presenting the primary amine had not been designed or selected for catalysis.

In the context of catalytic nucleic acids, a related question might be raised, namely whether the absence of a primary amine might explain the lack of a Schiff base activity. Could nucleobase nitrogens, which are known to react with aldehydes, also exhibit the ability to form a Schiff base so as to activate a carbonylic substrate such as an abasic site?[12] No naturally occurring ribozyme has been found to use a nucleobase nitrogen as a nucleophile for any reaction type. SELEX and related methodologies[13] have enabled the discovery of numerous nucleic acid catalysts that are not found in nature.[14] In terms of addressing the chemistry of nucleobase nitrogens, combinatorial selection yielded an RNA strand with enhanced nucleobase nucleophilicity; it efficiently alkylates itself at the N7 of a single G, without turnover.[15] Other key studies have revealed enhanced nucleophilic activity of synthetically appended 5′-amines (as well as 5′-thiols) in terms of acylation and alkylation.[16] The resulting self-modifying "catalysts" are generally incapable of turnover,[17] except in certain cases where they have been re-engineered to attain this attribute.[18]

The long-range goal of converting self-modifying nucleic acids into species with catalytic turnover has general implications with respect to nucleophilic enhancement, and more specifically to the nucleophilic aspect of covalent Schiff base catalysis examined in this study. In addressing this long-range goal, one might reserve the term "covalent catalysis" to describe cases whereby the catalyst engages substrate in a transient covalent intermediate that (a) cannot be observed in the uncatalyzed reaction, (b) lowers the activation energy or enhances the specificity of a second step, and (c) ultimately decomposes to regenerate active catalyst.[19,20] Only a few ribozymes are known to engage substrate in a transient covalent intermediate that decomposes under steady state to give product and regenerate free catalyst.[21,22] Covalent catalysis of this kind has been limited to ribozymes synthetically appended with thiol nucleophiles and has yet to be observed in DNAzymes. Moreover, covalent Schiff base catalysis (with or without turnover) has yet to be observed in a catalytic nucleic acid of any kind.

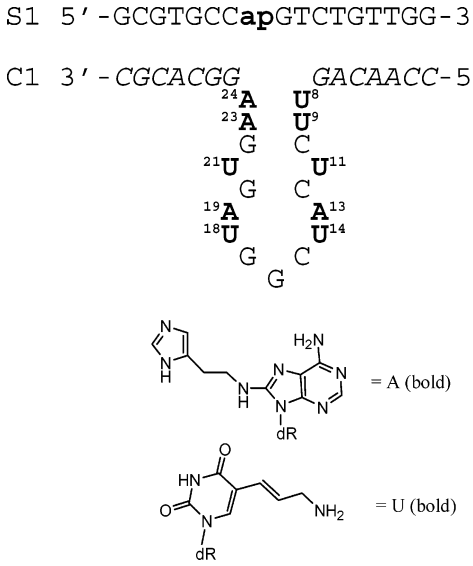
Combinatorial selection of DNAzymes from modified dNTPs appended with cationic amines, imidazoles, or both offers new prospects for catalyst design. Such modified DNAzymes (and ribozymes) might overcome some of the inherent functional and chemical limitations of their unmodified congeners by accessing functionalities generally characteristic of proteins.[23] A few DNAzymes[24] and aptamers[25] presenting at least one of these synthetic functionalities have been reported. To date, none exhibits covalent Schiff base catalysis.

As part of an ongoing interest in chemically modified DNAzymes, we undertook a preliminary investigation into the chemical and kinetic aspects of a chemically modified DNAzyme that promotes sequence-specific strand scission of a complementary DNA oligonucleotide containing an AP (apurinic/apyrimidinic) site. This activity is observed in a DNAzyme identified in a combinatorial selection for M^{2+}-independent RNaseA activity in which 8-histaminyl-dATP replaced dATP and 5-aminoallyl-dU replaced TTP.[26] From this family, several sequences were cloned and the sequence **C1**, 5′-*CCAACAG*-**UU**C**U**C**AU**CCG**UAGU**G**AA***GGCACGC*-3′, was resynthesized on solid phase (bold type indicates a duly modified nucleotide).[27]

(11) Manoharan, M.; Andrade, L. K.; Cook, P. D. *Org. Lett.* **1999**, *1*, 311−4.
(12) (a) McGhee, J. D.; Von Hippel, P. H. *Biochemistry* **1975**, *14*, 1281−96. (b) Wang, M.; McIntee, E. J.; Cheng, G.; Shi, Y.; Villalta, P. W.; Hecht, S. S. *Chem. Res. Toxicol.* **2001**, *14*, 423−30.
(13) (a) Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505−10. (b) Ellington, A. D.; Szostak, J. W. *Nature* **1990**, *346*, 818−22. (c) Robertson, D. L.; Joyce, G. F. *Nature* **1990**, *344*, 467−8. (d) Breaker, R. R.; Joyce, G. F. *Chem. Biol.* **1994**, *1*, 223−9.
(14) (a) Unrau, P. J.; Bartel, D. P. *Nature* **1998**, *395*, 260−3. (b) Jenne, A.; Famulok, M. *Chem. Biol.* **1998**, *5*, 23−34. (c) Sun, L. L.; Cui, Z. Y.; Gottlieb, R. L.; Zhang, B. L. *Chem. Biol.* **2002**, *9*, 619−28. (d) Huang, F. Q.; Bugg, C. W.; Yarus, M. *Biochemistry* **2000**, *39*, 15548−55. (e) Travascio, P.; Bennet, A. J.; Wang, D. Y.; Sen, D. *Chem. Biol.* **1999**, *6*, 779−87. (f) Li, Y. F.; Sen, D. *Biochemistry* **1997**, *36*, 5589−99. (g) Conn, M. M.; Prudent, J. R.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 7012−3. (h) Seelig, B.; Jaschke, A. *Chem., Biol.* **1999**, *6*, 167−76. (i) Sheppard, T. L.; Ordoukhanian, P.; Joyce, G. F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7802−7. (j) Ordoukhanian, P.; Joyce, G. F. *J. Am. Chem. Soc.* **2002**, *124*, 12499−506. (k) Carmi, N.; Shultz, L. A.; Breaker, *Chem. Biol.* **1996**, *3*, 1039−46. (l) Roth, A.; Breaker, R. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6027−31.
(15) Wilson, C. W.; Szostak, J. W. *Nature* **1995**, *374*, 777−82.
(16) Sengle, G.; Eisenfuhr, A.; Arora, P. S.; Nowick, J. S.; Famulok, M. *Chem. Biol.* **2001**, *8*, 459−73. (b) Jadhav, V. R.; Yarus, M. *Biochemistry* **2002**, *41*, 723−9. (c) Kumar, R. K.; Yarus, M. *Biochemistry* **2001**, *40*, 6998−7004. (d) Jenne A.; Famulok, M. *Chem. Biol.* **1998**, *5*, 23−34.
(17) Reviewed in: (a) Breaker, R. R. *Chem. Rev.* **1997**, *97*, 371−90. (b) Jaschke, A.; Seelig, B. *Curr. Op. Chem. Biol.* **2000**, *4*, 257−62.
(18) Zaug, A. J.; Been, M. D.; Cech, T. R. *Nature* **1986**, *324*, 429−33.
(19) The distinction is perhaps a subtle one. Self-modifying sequences that have been selected for enhanced nucleophilicity have been described as "catalysts". If turnover is a desirable quality to be pursued in the development of a catalyst, then in some cases it can be "engineered" by divorcing the nucleophile from the catalytic domain to provide for a catalyzed bimolecular condensation between nucleophile and electrophile. Consequent to the engineering step that provides for turnover, the term "nucleophilic catalysis" loses pertinence because the nucleophile consequently resides on the newly appointed substrate and no longer on what had been the self-modifying strand. We would like to distinguish cases of enhanced nucleophilicity that result in irreversible self-modification from cases where a nucleophilic attack precedes a second critical bond making/breaking step and where the covalent adduct that forms is used to lower the activation energy in this second step. Although the issue of turnover is not addressed in this description, its relation to the definition of catalysis is generally considered integral.
(20) An exception to this statement would be a catalyst that used an unbound amino cofactor to promote a Mannich or Stork enamine reaction involving a resonance-stabilized anion but where the cofactor is released from the product and recycled (turnover).
(21) Illangasekare, M.; Yarus, M. *RNA* **1999**, *5*, 1482−9.
(22) Although unexamined in ref 16c, the thioester of a self-acylating ribozyme will hydrolyze to regenerate free thiol and catalyze acyl transfer with turnover.
(23) (a) Benner, S. A.; et al. *Pure Appl. Chem.* **1998**, *70*, 263−6. (b) Sakthivel, K.; Barbas, C. F. *Angew. Chem., Int. Ed.* **1998**, *37*, 2872−5. (c) Perrin, D. M.; Garestier, T.; Helene, C. *Nucleosides Nucleotides* **1999**, *18*, 377−91. (d) Matulic-Adamic, J.; Daniher, A. T.; Karpeisky, A.; Haeberli, P.; Sweedler, D.; Beigelman, L. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1299−302. (e) Vaish, N. K.; Fraley, A. W.; Szostak, J. W.; McLaughlin, L. W. *Nucleic Acids Res.* **2001**, *28*, 3316−22. (f) Sawai, H.; Ozaki, A. N.; Satoh, F.; Ohbayashi, T.; Masud, M. M.; Ozaki, H. *Chem. Commun.* **2001**, *24*, 2604−5. (g) Gourlain, T.; Sidorov, A.; Mignet, N.; Thorpe, S. J.; Lee, S. E.; Grasby, J. A.; Williams, D. M. *Nucleic Acids. Res.* **2001**, *29*, 1898−905. (h) Thum, O.; Jäger, S.; Famulok, M. *Angew. Chem., Int. Ed.* **2001**, *40*, 3990−3. (i) Held, H. A.; Benner, S. A. *Nucleic Acids Res.* **2002**, *30*, 3857−69. (j) Held, H. A.; Roychowdhury, A.; Benner, S. A. *Nucleosides, Nucleotides, Nucleic Acids* **2003**, *22*, 391−404. (k) Kuwahara, M.; Takahata, Y.; Shoji, A.; Ozaki, A. N.; Ozaki, H.; Sawai, H. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3735−8. (l) Schoetzau, T.; Langner, J.; Moyroud, E.; Rohl, I.; Klussmann, S.; Vonhoff, S. *Nucleosides, Nucleotides, Nucleic Acids* **2003**, *22*, 1293−6. (m) Schoetzau, T.; Langner, J.; Moyroud, E.; Roehl, I.; Vonhoff, S.; Klussmann, S. *Bioconj. Chem.* **2003**, *14*, 919−26.
(24) (a) Teramoto, N.; Imanishi, Y.; Ito, Y. *Bioconj. Chem.* **2000**, *11*, 744−8. (b) Wiegand, T. W.; Janssen, R. C.; Eaton, B. E. *Chem. Biol.* **1997**, *4*, 675−83. (c) Santoro, S. W.; Joyce, G. F.; Sakthivel, K.; Gramatikova, S.; Barbas, C. F. *J. Am. Chem. Soc.* **2000**, *122*, 2433−9. (d) Perrin, D. M.; Garestier, T.; Helene, C. *J. Am. Chem. Soc.* **2001**, *123*, 1556−63. (e) Tarasow, T. M.; Tarasow, S. L.; Eaton, B. E. *Nature* **1997**, *389*, 54−7. (f) Sidorov, A. V.; Grasby, J. A.; Williams, D. M. *Nucleic Acids Res.* **2004**, *32*, 1591−1601.
(25) (a) Battersby, T. R.; Ang, D. N.; Burgstaller, P.; Jurczyk, S. C.; Bowser, M. T.; Buchanan, D. D.; Kennedy, R. T.; Benner, S. A. *J. Am. Chem. Soc.* **1999**, *121*, 9781−9. (b) Vaish, N. K.; Larralde, R.; Fraley, A. W.; Szostak, J. W.; McLaughlin, L. W. *Biochemistry* **2003**, *42*, 8842−51.
(26) Perrin, D. M.; Garestier, T.; Helene, C. *J. Am. Chem. Soc.* **2001**, *123*, 1556−63.

This DNAzyme contains a putative catalytic domain (underlined) and substrate binding guide arms (italics) and is also presented in Scheme 1. No divalent metal cation is required for APE activity. Instead, the APE activity appears to involve covalent Schiff base catalysis. The results herein suggest that a primary amine may be vital for expanding the catalytic repertoire of nucleic acids, not only to surmount the well-appreciated electrostatic deficiency but to deliver covalent Schiff base catalysis as well.

Scheme 1. 31-mer DNAzyme Associated with Abasic Substrate



Materials and Experimental Methods

General Considerations. All syntheses were carried out with the exclusion of bright light under an argon atmosphere in flame-dried glassware. Anhydrous reagents and solvents were prepared according to standard procedures (Perrin and Armarego). Flash chromatography was carried out using Silica gel 60, 230−400 mesh, supplied by E. Merck Co. Silica gel 60 F_{254} on aluminum sheets (E. Merck, type 5554) was used for TLC analysis. Nuclear magnetic resonance spectra (^{1}H and ^{31}P) were obtained on a Bruker AV-300 or Bruker AV-400 instrument. Solvent, signal multiplicity, coupling constants, and integration ratios are indicated in parentheses. An external standard of 85% H_3PO_4 was used for ^{31}P NMR. (ESI) MS were obtained on a Bruker Esquire-LC instrument.

Synthesis and Characterization of 6-*N*-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-8-(methylamino)-2′-deoxyriboadenosine-3′-*O*-(*N*,*N*-diisopropylamino-*β*-cyanoethoxy)phosphoramidite. The protocol used was adapted from antecedent work.[28] Protected 8-methylamino-dA (0.48 g, 0.70 mmol) was dried overnight by coevaporation from a 2 mL solution of 10% pyridine in CH_2Cl_2. The sample was redissolved in 13 mL of CH_2Cl_2, and *N*,*N*-diisopropylethylamine (freshly distilled over CaH_2) (219 μL, 1.41 mmol) was added. After cooling to −78 °C, 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite (233 μL, 1.05 mmol) was added dropwise. After 5 min, the clear colorless solution was allowed to warm to room temperature and was stirred continuously for 6 h. The reaction was quenched by addition of MeOH (0.1 mL), diluted in CH_2Cl_2, and washed with 10% aqueous sodium bicarbonate. The organic layer was dried with sodium sulfate, filtered, and concentrated under reduced pressure to afford a white foam. The foam was dissolved in a minimal amount of EtOAc (2 mL) and precipitated by the addition of of cold hexane (50 mL). The precipitate was centrifuged, and the supernatant was removed. The further addition of approximately 5 volumes of hexane to the supernatant afforded a second crop of precipitate. The precipitates were combined and further purified by silica gel chromatography and eluted with 9.2:9.2:1:0.6 hexanes/EtOAc/MeOH/Et_3N to afford product (0.43 g, 70%) as a white foam. ^{1}H NMR ($CDCl_3$, 400 MHz): 8.88 (s, 1H), 8.50 (s, 1H), 7.94 (d, J = 7.5 Hz, 2H), 7.51 (t, J = 7.3 Hz, 1H), 7.43 (t, J = 7.3 Hz, 2H), 7.38−7.20 (m, 9H), 6.82 (m, 4H), 6.55 (m, 1H), 5.96 (m, 1H) 4.78 (m, 1H), 4.19 (m, 0.5H), 4.14 (m, 0.5H), 3.76 (s, 3H), 3.76 (s, 3H), 3.86−3.44 (m, 5H), 3.36 (m, 1H), 2.88 (m, 1H), 2.72 (m, 1H), 2.59 (t, J = 6.2 Hz, 1H), 2.46 (dd, J = 13.6, 5.6 Hz, 0.5H), 2.39−2.30 (m, 3.5H), 1.24−0.98 (m, 12H). ^{31}P NMR ($CDCl_3$): 149.77, 149.22 (external standard of 85% H_3PO_4). MS (ESI): 925.5 $(M + K)^+$, 909.5 $(M + Na)^+$, 887.6 $(M + H)^+$.

Oligonucleotide Synthesis and Purification. Substrate oligonucleotides (**S1**, **S2**) were synthesized by the NAPS (Nucleic Acid Peptide Sequencing) unit of UBC, using standard automated solid-phase methods on Applied Biosystems DNA synthesizers and deprotected with concentrated NH_4OH at 65 °C overnight. **S1**, a complementary substrate, and **S2**, an inverted substrate, were synthesized as precursors 5′-d(GCGTGCCUGTCTGTTGG)-3′ and 5′-d(GGTTGTCTGUCCGTGCG)-3′, respectively, that contained a single dU, which could be depyrimidinated by uracil DNA glycosylase to generate suitable substrates with abasic sites. Modified oligonucleotides (catalysts **C1** and **C2**) were synthesized from standard dG, dC, dA, and dT precursor phosphoramidites. Where indicated, 5-aminoallyl-dU[29] was used in place of dT for the synthesis of **C1** and **C2** and either 8-histaminyl-dA[30] or 8-MeNH-dA, respectively, replaced dA in the sequence 5′-d(*CCAACAG***UUCUCAU***CCG***UAGUGAA***GGCACGC*)-3′ (bold U indicates modification with aminoallyl-dU, bold A indicates either 8-histaminyl-dA for **C1** or 8-methylamino-dA for **C2**, underlined represents the putative catalytic motif, italics indicate no modification and represent the guide sequences). The synthesis was carried out by Dr. Richard Pon at the University of Calgary Core DNA and Protein Service Facility. In addition, **C0**, an entirely unmodified oligonucleotide of sequence 5′-d(CCAACAGTTCTCATCCGTAGTGAAGGCACGC)-3′ was also synthesized for control experiments. Samples were deprotected with concentrated NH_4OH at 65 °C, resolved by 15−20% 7 M-Urea PAGE (29:1 monomer/bis acrylamide), identified by UV-shadowing, eluted into aqueous 1% $LiClO_4$ 7 mM triethylamine, lyophilized, resuspended in 100 μL of H_2O, precipitated with 10 volumes of ethanol, and desalted over a G25 Sephadex spin column equilibrated with water.

Substrate Preparation. 5′-End labeling was effected with ATPγ^{32}P (10−20 μCi, Perkin-Elmer) and T4 polynucleotide kinase (1−2 units, New England Biolabs) for 45 min at 37 °C. 3′-End labeling was effected with ddATPα^{32}P (10−20 μCi, Amersham) and terminal transferase (1−2 units, New England Biolabs) for 1−2 h at 37 °C in the presence of $CoCl_2$ (New England Biolabs). Following ^{32}P-labeling, oligonucleotides were desalted over G25 spin columns and purified by 20% 7 M Urea D-PAGE. A short exposure with markers was used to identify the radiolabeled oligonucleotides, which were then eluted from the gel as before. To prepare abasic substrates, purified precursor **S1** and **S2** oligonucleotides, either in chemical or radiochemical quantities, were incubated for 2 h at 37 °C with uracil DNA glycosylase (≥1 unit per 100 pmol substrate, New England Biolabs) in 50 mM HEPES (pH 7.0), 5.0 mM NaCl. Samples were extracted with phenol/chloroform (1:1, v/v, Sigma) and desalted with a G10 spin column. The relative instability of the abasic site dictated immediate use following substrate preparation (the abasic substrate was found to be stable (>90%

(27) Lermer, L.; Roupioz, Y.; Ting, R.; Perrin, D. M. *J. Am. Chem. Soc.* **2002**, *124*, 9960−1.
(28) (a) Prakash, T. P.; Kumar, R. K.; Ganesh, K. N. *Tetrahedron* **1993**, *49*, 4035−50. (b) Lermer, L.; Hobbs, J.; Perrin, D. M. *Nucleosides, Nucleotides, Nucleic Acids* **2002**, *21*, 651−64.
(29) (a) Cook, A. F.; Vuocolo, E.; Brakel, C. L. *Nucl. Acids Res.* **1988**, *16*, 4077−95. (b) Bergstrom, D. E.; Ruth, J. L. *J. Carbohydr. Nucleosides Nucleotides* **1977**, *4*, 257−69.
(30) Lermer, L.; Hobbs, J.; Perrin, D. M. *Nucl., Nucl., & Nucl. Acids* **2002**, *21*, 651−64.

remaining) during a period of 2−5 days and not amenable to either freeze−thawing or concentration by lyophilization). Successful abasic site conversion was verified for each experiment by treatment of a sample with piperidine (10 vol %) at 95 °C for 15 min and resolving by 20% denaturing PAGE.

General Procedure for Kinetic Experiments. All kinetic experiments were layered with mineral oil to prevent nonspecific binding and evaporation in 600 μL Eppendorf PCR tubes. Single turnover assays were done by preparing a cocktail containing the catalyst at twice the desired catalyst concentration (2×) in the cleavage buffer (50 mM Tris HCl pH 7.5, 1 mM EDTA, 200 mM NaCl). A trace (<2 nM) of the 5′- or 3′-labeled substrate was also brought up in 1× cleavage buffer to provide a substrate cocktail. Substrate and catalyst cocktails were incubated at the desired reaction temperature in a Julabo F-10 temperature-controlled water bath for a minimum of 1.5 h prior to mixing. Reactions were initiated by the addition of substrate to catalyst. Aliquots were taken out at desired times, quenched with 95% formamide and 1 mM EDTA, and resolved on a 20% D-PAGE gel without heating, unless otherwise noted. Results from PAGE assays were quantified by autoradiography using a Typhoon Phosphorimager (Molecular Dynamics−Pharmacia). Data were worked up using Sigma Plot software.

Single-Turnover Experiments. Substrate cocktails containing AP-site DNA (0.25 μM final concentration) in reaction buffer (500 mM NaCl, 50 mM Tris HCl pH 7.5, 1 mM EDTA) were prepared and divided into individual tubes. A "2×" solution of catalyst (5 μM) was prepared so that a final concentration of 2.5 μM could be attained when 5 μL of catalyst (i.e., **C0**, **C1**, or **C2**) was added to tubes containing 5 μL 2× buffer salts and trace quantities of radiolabeled AP-substrate (**S1** or **S2**). Reactions were quenched at the stated times by adding 15 μL of formamide/dyes (95% formamide, 25 mM EDTA + XC/BΦB), loaded without heating, and resolved by 20% 7 M Urea D-PAGE. Cleavage controls with piperidine (treated at 1 M at 95 °C for 15 min, then lyophilized and resuspended in formamide) were included for reference.

Sizing Experiments. Single-turnover cleavage reactions were run either with 5′-labeled **S1** and unlabeled **C1** or with 5′-labeled **C1** and unlabeled **S1**. A reference Maxam-Gilbert G+A ladder was run on an oligonucleotide of sequence 5′-^{32}P-T$_9$GAGCTCGCGGGG CGTGCCTTCACTACGGATGAGAACTGTTGGTAGGGCCCAA-CAGAGGGCACGCTCGTGTCGT-3′, depurinated with a 1 M piperidine formate stock solution (formic acid: 0.05%, piperidine: 0.005%), lyophilized, and heated at 95 °C for 30 min in 1 M piperidine.

Rescue with 4-Methylimidazole. A solution of 200 mM 4-methylimidazole was brought to pH 7.5 with HCl. Aliquots (5 μL) of this solution were added to tubes for rescue experiments, which were then evaporated to dryness. Substrate and catalyst cocktails were made as before to achieve final reaction concentrations of 0.25 and 2.5 μM, respectively, but with phosphate buffer (500 mM NaCl, 50 mM $NaHPO_4$ pH 7.4, 1 mM EDTA). Aliquots were distributed to tubes (including those with 4-methylimidazole) to achieve total reaction volumes of 5 μL. Reactions were quenched with 15 μL formamide (95%)/25 mM EDTA at the given times.

Multiple-Turnover Experiment. Conditions of this experiment were identical to those of the single-turnover experiment except that either 5 or 10 times excess of substrate was used, relative to catalyst concentration (i.e., 1.25 or 2.5 μM substrate, 0.25 μM catalyst). To obtain this quantity of substrate, unlabeled **S1** containing an AP-site was used in conjunction with labeled **S1** to prepare a substrate cocktail with 50−100 kcpm. To ensure that both the labeled and unlabeled species were converted, unlabeled substrate precursor was mixed with a trace of labeled substrate precursor, the entire quantity of which was treated with UDG. The quantity of conversion was confirmed by piperidine cleavage and analyzed by minigel PAGE.

Thermocycling Experiments. Using the same reaction conditions as above for multiple-turnover experiments, a drop of mineral oil was laid over the reaction and then cycled in a PCR thermocycler 30 times from 15 °C (0.25 min) to 22 °C (10 min) to 75 °C (1 min). Reactions were quenched by the addition of formamide (95%)/25 mM EDTA + XC/BΦB dyes for resolution by 20% Urea-PAGE. A control without **C1** was also run.

Determination of Kd (dissociation constant between substrate and C0, C1, and **C2**). Gel-shift assays performed at 22−24 °C were used to determine equilibrium dissociation constants of substrate and catalyst oligonucleotides by using a trace amount of radiolabeled substrate (either 3′- or 5′-labeled) and increasing concentrations of **C1** that had been dried down and resuspended in 10 μL of 200 mM NaCl, 50 mM Tris HCl pH 7.5, and 1 mM EDTA containing trace amounts of 5′-labeled **S1**.[31] After 5 min, 1 μL of 70% sucrose and blue dyes was added to the sample, which was then loaded into a 20% native polyacrylamide gel (29:1 monomer:bis) containing 50 mM Tris HCl and 200 mM NaCl, with 50 mM Tris HCl, 200 mM NaCl running buffer. To compare the *relative* K_d's of **C0** (the unmodified sequence), **C1** (catalyst), and **C2** (catalytically impaired), a DNA oligonucleotide of sequence 5′-d(GCGTGCCCGTCTGTTGG)-3′ was used for convenience. The catalyst (at varying concentrations of **C0**, **C1**, or **C2**) was incubated for 5 min before loading in a 20% 29:1 bis:acrylamide gel. All gels and equipment were equilibrated at the running temperature prior to loading. Visualized on a Molecular Dynamics Typhoon phosphorimager. Polygons were drawn around distinct bands, and fractions of product formation (e.g., gel-shifted band, liberated substrate or product) were calculated, on the basis of volume intensities within the polygons. The fractions of bound substrate were determined and fitted to the hyperbola:

$$[\mathrm{ES}^*] = \frac{[\mathrm{E}]}{K_d + [\mathrm{E}]} \tag{4}$$

where [ES*] is the fraction of labeled substrate bound to catalyst at the different catalyst concentrations, [E]. K_d is the equilibrium constant for the catalyst substrate complex. The use of 5′-d(GCGTGCCCGTCT-GTTGG)-3′ gave K_d values for **C1** that were within 30% of those reported for the abasic substrate.

Determination of Dissociation Rate Constants of Substrate (S1) Oligonucleotide with C1. Chase experiments were performed in the following manner. A cocktail of 15 μL containing 2 μM catalyst C1 in 1× buffer was rapidly mixed with 15 μL of 1× buffer containing <2 nM of the 3′-labeled **S1** to give a catalyst concentration of 1 μM (>100 times the K_d for **C1**−**S1** interaction determined by simple gel retardation analysis) and incubated at 24 °C for an additional 3 min before the chase was added. For the chase, 30 μL of 25 μM unlabeled substrate **S1** in 1× cleavage buffer was then added to the above solution (28.5 μL) containing the catalyst and radiolabeled trace substrate. At various time points over a period of 55 min, 3 μL aliquots were removed following the chase and placed into ice-cold tubes containing 3 μL of 10% sucrose bromophenol blue and xylene cyanol. These aliquots were placed on ice until the completion of the experiment. The samples were loaded at 4 °C into a 20% 29:1 monomer/bisacrylamide gel containing 50 mM Tris HCl and 200 mM NaCl (dimensions 150 mm × 150 mm × 1 mm) with 50 mM Tris HCl and 200 mM NaCl electrophoresis buffer and resolved at 10 W constant until the bromophenol blue had migrated 1 cm. The gel had been precooled and run at 4 °C. To ensure that dissociation was effectively arrested at 4 °C and did not continue during the 55 min chase, the entire set of tubes was stored at 4 °C for an additional 1080 min (total variance of 1080−1135 min) and rerun reproducibly.

Following phosphorimaging, first-order rate constants (k_{obs}) were obtained by fitting data to the single-exponential eq 1 using the Sigma Plot v7.101 data analysis program.

$$[\mathrm{P}]_t = [\mathrm{P}]_\infty e^{-k_{obs}t} \tag{1}$$

(31) Hertel, K. J.; Herschlag, D.; Uhlenbeck, O. C. *Biochemistry* **1994**, *33*, 3374−85.

where $[P]_t$ and $[P]_\infty$ are the fraction of product at time t and the end point, respectively, where k_{obs} is the first-order rate constant.

Trapping Experiments. Substrate (5′-labeled) and catalyst (**C1** and **C2**) cocktails were prepared as described for the single-turnover experiment. Final reaction concentrations: substrate 0.25 μM; catalyst 2.5 μM in reaction buffer (500 mM NaCl, 50 mM Tris HCl pH 7.5, 1 mM EDTA). Solutions of 500 mM of $NaBH_4$ and $NaCNBH_3$ were freshly prepared and added to the reaction tubes at the stated times for a final concentration of 50 mM reducing agent. Reactions were "quenched" at the stated times by adding 15 μL of formamide/dyes (95% formamide/25 mM EDTA/blue dyes). Samples were loaded without heating and resolved by 20% D-PAGE.

Cyanide Inhibition Studies by Native Gel Retardation. Twelve microliters of a "1.5×" cocktail that was 3.75 μM catalyst, 750 mM NaCl, 75 mM Tris HCl pH 7.5, and 1.5 mM EDTA, was distributed to tubes 1−8. Four microliters of water or 30 mM KCN was added to tubes where cyanide was to be absent or present, respectively. 3′-Labeled substrate (2 μL) in water was added to tubes either 12 or 24 h prior to the chase, bringing the final volume to 18 μL. Reaction proceeded on the bench at 22.5 °C. Tubes 5−8 received 2 μL of a chase solution that contained both 50 μM d(GCGTGCCCGTCTGTTGG) and 50 μM d(GTCTGTTGG) in 500 mM NaCl, 50 mM Tris HCl pH 7.5, and 1 mM EDTA. The reactions were then chased for 20 min to allow complete displacement of any noncovalently bound substrates with unlabeled substrates. Following the chase, all samples were placed at 0−4 °C. Following addition of 2 μL of 70% sucrose and resolving dyes (BΦB and XC), samples were loaded into a 20% nondenaturing polyacrylamide (29:1 monomer:bis) gel containing 200 mM NaCl, 50 mM Tris HCl pH 7.5, and 1 mM EDTA and run in the same buffer at 7.5 W for 2 h at 4 °C such that the BΦB had migrated 1−1.5 cm. To prepare 3′-labeled product, 2 μL of 3′-labeled substrate had been treated with 20 volumes of 1 M piperidine, lyophilized 3× from water, and resuspended in buffer and sucrose. For the catalyst−substrate complex control, **C1** and 3′-labeled **S1** were mixed 10 min prior to loading and not chased to provide a standard for where the noncovalently associated catalyst−substrate complex would migrate.

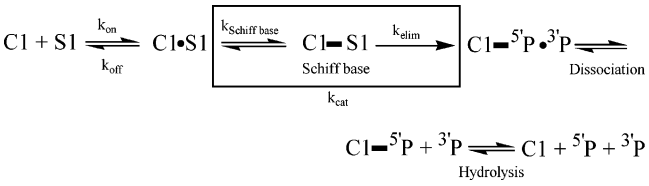
Control Experiments with Amino Acids and Unmodified Sequence. 3′-Labeled substrate (**S1**) was prepared in reaction buffer (500 mM NaCl, 50 mM Tris HCl pH 7.5, 1 mM EDTA) for a final concentration of 0.25 μM. Solutions of the amino acids lysine and histidine were prepared and added to the reaction tubes at concentrations of 250 μM, 1.5 mM, and 5 mM. In addition, a solution containing both lysine and histidine was made and added in the same way at final concentrations of 82.5, 150, and 500 μM (concentration of each amino acid). Reactions were quenched at the stated times by adding 15 μL of formamide dyes. Samples were loaded without heating and resolved by 20% D-PAGE.

Kinetic Framework

Based on the hypothesis of Schiff base-promoted APE activity, a kinetic framework was outlined as presented in Scheme 2: Substrate and catalyst associate via a noncovalent, sequence-specific interaction mediated by Watson−Crick base pairing. Subsequently, dehydration gives rise to a covalent Schiff base linkage between catalyst and substrate whereupon lyase activity results in strand scission. Hydrolysis of the remaining Schiff base-linked 5′-product regenerates free catalyst.

Within the kinetic framework of Schiff base catalysis lie two further possibilities concerning the relative rates of Schiff base formation and strand scission. If Schiff base formation is fast compared to strand scission, the Schiff base linkage to substrate should accumulate over time and would be resolved by denaturing PAGE provided that this covalent linkage is stable. If Schiff base formation is slow relative to strand scission, the covalently linked catalyst−substrate intermediate should not accumulate. Instead, a product of higher apparent molecular weight comprising a covalent linkage between 5′-product and catalyst would appear in a time-dependent manner at a rate that is consistent with that observed for strand scission.

Scheme 2. Kinetic Scheme for Schiff Base Formation and Cleavage of Substrate **S1** by Catalyst **C1** at 24 °C; $^{5'}$P: 5′ Oligonucleotide Product, $^{3'}$P: 3′ Oligonucleotide Product



The initial kinetic analysis presented herein is evaluated using single-turnover and pre-steady state conditions. Under these conditions, the overall rate of strand scission will be determined by at least three forward reactions: (i) substrate association, (ii) Schiff base formation, and (iii) elimination, and two back reactions: (i) dissociation and (ii) Schiff base hydrolysis. The 1,4 Michael addition that constitutes the third back reaction is ignored in this analysis because the forward reaction was found to proceed quantitatively under single turnover conditions.

Results

The kinetic framework was tested using a number of synthetic constructs that are summarized as follows. Along with **C1**, two other oligonucleotides, **C0** and **C2**, were synthesized with the same sequence as the functional catalyst **C1**. **C0** contained no modifications whatsoever, whereas **C2** contained the six 5-aminoallyl-dU's and four 8-methylamino-dA's in place of the 8-histaminyl-dA's. The replacement of 8-histaminyl-dA with 8-methylamino-dA was based on recent reports that 8-amino purines adopt considerably different conformations around the C1′-N9 glycosidic linkage and form particularly effective 3H-bond Hoogsteen base pairs with pyrimidines.[32] In this manner the imidazole was removed while preserving any structural aspects related to the 8-amino purine. A complementary substrate, **S1**. 5′-d(GCGTGCCapGTCTGTTGG)-3′, and an inverted substrate, **S2**, 5′-d(GGTTGTCTGapCCGTGCG)-3′, were generated by uracil-DNA glycosylase (UDG) action on precursors containing dU to give an abasic site denoted by "ap". Prior to UDG action, substrate precursors were selectively labeled either at the 5′-terminus using ATPγ^{32}P and T4-PNK or at the 3′-terminus using ddATPα^{32}P and TdT. With this labeling scheme, the fate of the 5′- and 3′-termini of the substrate could be monitored during the reaction. The inverted substrate **S2** was used to evaluate the sequence specificity for the reaction. Association and dissociation rate constants were determined from gel-retardation and pulse-chase reactions.

The preliminary observation, which demonstrated that **C1** could act as an APE, is shown in Figure 1, where **C1** completely cleaved 3′-labeled **S1** in a time-dependent manner within 25 h and yielded a product of lower apparent molecular weight (band I). Piperidine digestion of **S1** provided independent electrophoretic calibration for 3′-product migration in a denaturating polyacrylamide gel.

Various experiments were then run to address both the sequence specificity and the chemical basis of the observed

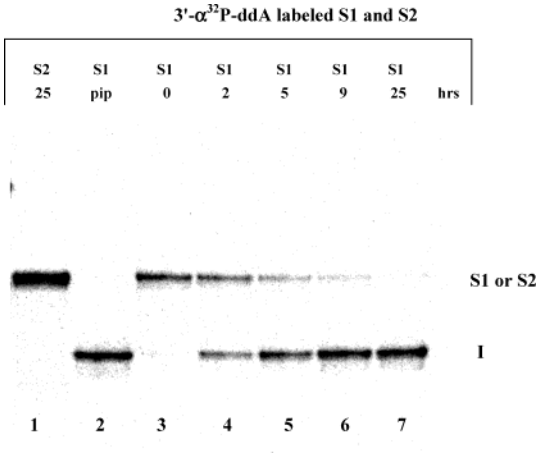


Figure 1. Lanes 1−7: 3′-labeled substrates, all reactions 10 μL of 2.5 μM catalyst (**C1**), 0.25 μM **S1** or **S2**, 24 °C, in 500 mM NaCl, 50 mM Tris HCl pH 7.5, 1 mM EDTA, quenched with 15 μL of formamide, no heat, and resolved by 20% denaturing urea-PAGE.

cleavage reaction. An inverted sequence **S2**, incapable of antiparallel association, was investigated and found to be resistant to any cleavage by **C1** (Figure 1, lane 1), suggesting a sequence-specific interaction based on Watson−Crick pairing.[33] Similar results were also observed in phosphate, cacodylate, and HEPES buffers (data not shown). To further substantiate that the strand scission was due to the sequence-specific interaction of the abasic site with *modified* DNA bases and not simply attributable to either buffer action alone or buffer action in conjunction with a gapped duplex, the same reaction was initiated on **S1** in the presence of buffer salts (Tris HCl) without (Figure 2, lanes 1−6) or with the unmodified analogue **C0** (Figure 2, lanes 7−12). Figure 2 clearly demonstrates that the

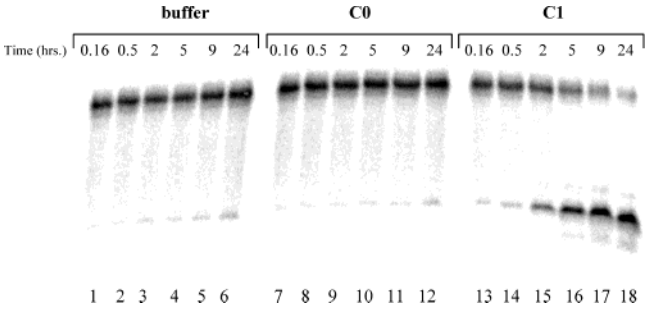


Figure 2. Lanes 1−18: 3′-labeled substrate d(GCGTGCCapGTCTGTT). Lanes 1−6: time course, buffer control. Lanes 7−12: time course **C0**, no modifications. Lanes 13−18: time course for **C1** (reproduction of Figure 1). All reactions: 10 μL, 2.5 μM catalyst (**C1** or **C0**), 0.25 μM substrate, 23−24 °C, in 500 mM NaCl, 50 mM Tris HCl pH 7.5, 1 mM EDTA, quenched with 15 μL of formamide, no heat, and resolved by 20% denaturing urea-PAGE.

synthetic functionalities found on **C1** are integral for strand scission (lanes 13−18). Furthermore, no rescue of activity was observed by lysine, histidine, or both, with or without **C0** (see Supporting Information). Thus, this DNAzyme cleaves an abasic site in a sequence-specific manner by virtue of synthetic functionalities that are involved in a reaction not generally accessible to abasic sites. A rate constant for the observed scission, $k_{cat-overall} \approx 6 \times 10^{-3}$ min^{-1}, was estimated from the 2 h half-point of the reaction and corresponds to a rate enhancement of ~100.[34] This rate constant is a composite value that is determined by the rate constants for the formation and destruction of any intermediates leading up to strand scission. To define the individual rate constants leading up to this irreversible event, a more detailed kinetic investigation was undertaken.

The first set of rate constants to be considered are those governing the formation and dissociation of a noncovalent complex between substrate and catalyst: k_{on} and k_{off}. In general, second-order association rate constants for various ribozymes and DNAzymes have been found to be in the range 10^6-10^7 M^{-1} s^{-1} such that when the catalyst concentration is >1 μM, a pseudo-first-order association rate constant with respect to substrate complexation is large.[35] In addition, when 10 or more base pairs are formed, K_d's are low and substrates are saturated. Nevertheless, k_{off} and K_d were determined experimentally, and k_{on} was calculated therefrom. A K_d for the **C1**−**S1** interaction was ascertained by gel-retardation assays. In this case, a trace amount of labeled **S1** (3′ or 5′) was incubated with increasing amounts of **C1** for 5 min (a period that was sufficiently short that no chemical reaction occurs) and then resolved by native 20% PAGE. The data were fitted to a hyperbola, and a K_d was calculated to be approximately 2 nM (for 3′-labeled) and 9 nM (for 5′-labeled), with this difference attributed to the extra ddA at the 3′-terminus (data in Supporting Information).

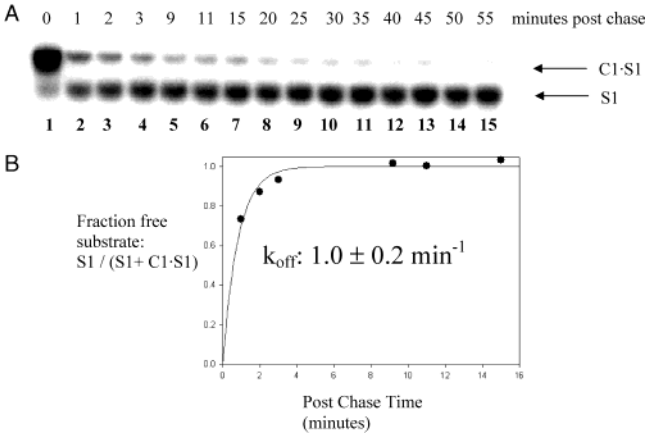


Figure 3. (A) Chase data over a period of 55 min (lanes 1−15). **(**B) First-order fit (rise to max.) over the first 15 min of appearance of substrate band.

A pulse-chase technique was used to measure the dissociation constant for the **C1**−**S1** interaction. In summary, a trace amount of 3′-labeled **S1** was mixed for 5 min with 1 μM **C1** and then chased with an equal volume of 25 μM unlabeled **S1**. Aliquots from this were removed at various time points over a period of 55 min, stored at 4 °C, and resolved by native PAGE at 4 °C (Figure 3A). The data were then fitted to a first-order rate expression and k_{off} was calculated to be 1 min^{-1} (Figure 3B). Using this dissociation rate constant and an average K_d of 5

(32) (a) Evans, F. E.; Wright, J. M. *Biochemistry* **1980**, *19*, 2113−7. (b) Soliva, R.; Garcia, R. G.; Blas, J. R.; Eritja, R.; Asensio, J. L.; Gonzalez, C.; Luque, F. J.; Orozco, M. *Nucl. Acids. Res.* **2000**, *28*, 4531−9. (c) Cubero, E.; Aviñó, A.; de la Torre, B. G.; Frieden, M.; Eritja, R.; Luque, F. J.; González, C.; Orozco, M. *J. Am. Chem. Soc*. **2002**, *124*, 3133−42.

(33) Piperidine treatment confirmed 100% abasic site conversion on **S2**.

(34) The rate enhancement is estimated to be 112 from the k_{uncat} 5.3 × 10^{-5} min^{-1} (0.1 M KCl, 0.05 M Hepes-KOH, 0.5 mM EDTA pH 7.4, extrapolated from 70 °C back to 22 °C) measured by Lindahl and Andersson, ref 6. It is difficult to consider rate enhancements of two reactions that do not operate via the same mechanism.

(35) (a) Santoro, S.; Joyce, G. F. *Biochemistry* **1998**, *37*, 13330−42. (b) Shih, I.-h.; Been, M. D. *Biochemistry* **2000**, *39*, 9055−66.

nM, a value of 2×10^8 M^{-1} min^{-1} for a second-order association rate constant, k_{on}, is estimated. This value is consistent with association rate constants found for other catalytic nucleic acids. Consequently, at 2.5 μM **C1** the pseudo-first-order association rate constant for substrate complexation is estimated to be approximately 500 min^{-1}. Dissociation constants of 60 and 30 nM for **C0** and **C2**, respectively, were also ascertained (data not shown) to ensure that the trace quantities of **S1** were saturated at 2.5 μM **C0** (Figure 2) or 2.5 μM **C2** (Figure 8A,B *vide infra*). Thus any lack of activity with **C0** and **C2** could not be attributed to partial saturation of **S1**. Having established that at 2.5 μM **C1** the association with substrate is rapid and saturating, the kinetic analysis simplifies to a first-order analysis where the **C1**−**S1** complex reacts in unimolecular-like fashion.[36]

At this juncture we entertained two hypotheses regarding the mechanism that would lead to this cleavage: (1) Cleavage was direct, in which case Schiff base catalysis was not operative, and (2) a Schiff base between catalyst and substrate represented a *bona fide* intermediate in the course of an E1cB/β elimination that enhanced the catalyzed rate for strand scission. Both hypotheses lead to predictions of what should be observed in terms of product formation. For instance, if Schiff base catalysis is not operative (or if 5′-product hydrolysis is fast), a 7 nucleotide product should be observed when using a 5′-labeled substrate. No such product was observed. Instead, **C1** reacted with 5′-labeled **S1** to give a species with a higher apparent molecular weight (band II) that appeared under denaturing conditions in a time-dependent manner (Figure 4, lanes 3−7). The formation of band II was 50% complete in 5 h, and its rate of formation was thus comparable (within a factor of 2.5) to that of lyase activity. This discrepancy may be ascribed to slightly different substrate properties that relate to the nature of the label; an extra dA at the 3′-terminus may have different properties compared to a 5′-phosphate. Similar effects were seen with respect to K_d values that varied 4-fold. As with the 3′-labeled substrate, this reaction was also found to be sequence specific; the inverted substrate **S2** gave no reaction (Figure 4, lane 1).

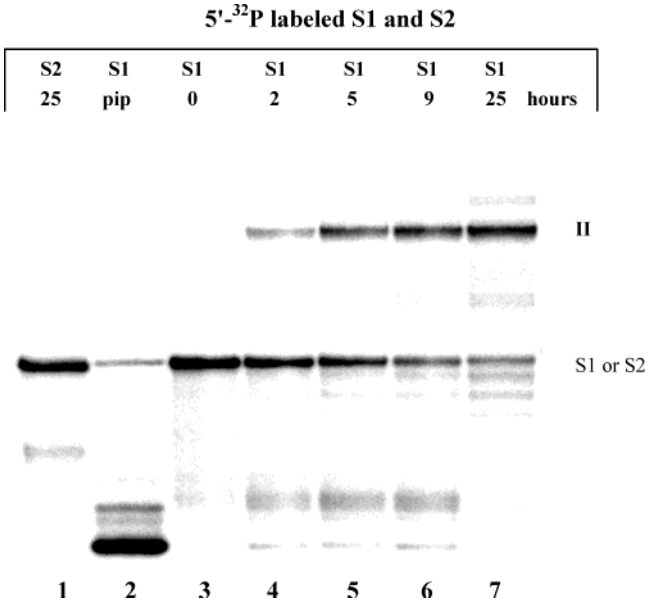


Figure 4. Lanes 1−7: 5′-labeled **S1** or **S2**. Lane 1: 25 h incubation with inverted **S2**. Lane 2: piperidine treatment on **S1**. Lanes 3−7: time course for Schiff base formation. Reaction proceeded at 24 °C, in 500 mM NaCl, 50 mM Tris HCl pH 7.5, 1 mM EDTA, quenched with 15 μL of formamide, no heat, and resolved by 20% denaturing urea-PAGE.

The appearance of a faster migrating band with a 3′-labeled substrate and a retarded band with a 5′-labeled substrate would constitute *prima facie* evidence of a Schiff base-promoted elimination reaction. The gel-shifted band seen only with 5′-labeled substrate under denaturing conditions led us to address several questions: (i) What is the nature of the species represented by band II in Figure 4? (ii) Does this band represent a Schiff-base linkage, and if so, is this linkage between **C1** and substrate or between **C1** and product? (iii) If this linkage was to 5′-product (as suggested by the absence of any such band when using 3′-labeled substrate), could a Schiff base linkage between **C1** and 5′-labeled substrate be trapped by $NaCNBH_3$ at early and late time points, respectively? Answering these questions would provide further characterization of this activity.

In addressing the chemical stability of band II, heat treatment was found to completely destroy the linkage and give rise to a product that moved slightly differently from a band that was derived from piperidine treatment (see Supporting Information). The electrophoretic difference between the heat-treated product and piperidine-treated substrate suggests different 3′-termini, namely, an α,β-unsaturated aldehyde and a phosphate, respectively. In contrast, borohydride treatment rendered band II completely stable to heat treatment (see Supporting Information) as well as to hot piperidine treatment (data not shown). Several additional experiments were then undertaken to suggest that band II in Figure 4 represented a Schiff base linkage to 5′-product, and not 5′-labeled substrate. In addition, it was important to demonstrate that band II was not only of a higher apparent molecular weight than **C1** but contained **C1**. To demonstrate this, 5′-labeled **S1** was allowed to react with unlabeled **C1** under the same conditions that 5′-labeled **C1** was allowed to react with unlabeled substrate. Irrespective of whether labeled substrate or labeled catalyst was used, a band of identical electrophoretic mobility appeared, suggesting that band II also comprised **C1**. The difference between band II and labeled catalyst was approximately 5 nucleotides, as gauged by a G+A sequencing ladder that provided independent reference of molecular weight. This is shown in Figure 5.

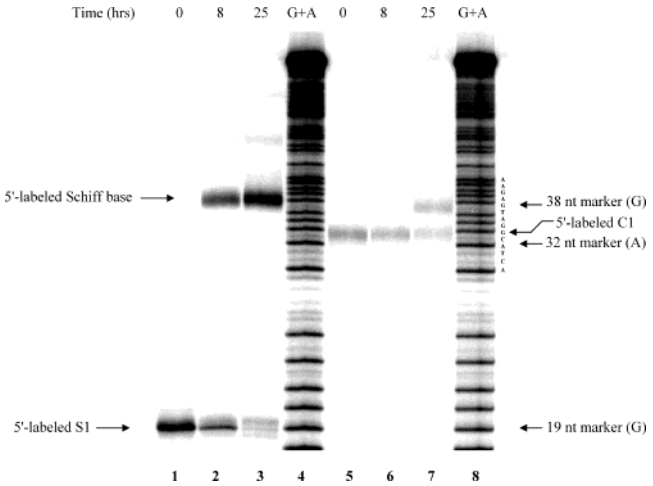


Figure 5. Lanes 1−3: 5′-labeled substrate reacted with excess **C1** (2.5 μM) at 0, 8, and 25 h, respectively. Lane 4: Maxam−Gilbert G+A sequencing ladder for molecular weight reference. Lanes 5−7: 5′-labeled **C1** (2.5 μM) and an equivalent of unlabeled substrate. Lane 8: Maxam−Gilbert G+A sequencing ladder for molecular weight reference. Reaction proceeded at 24 °C, in 500 mM NaCl, 50 mM Tris HCl pH 7.5, 1 mM EDTA, quenched with 15 μL of formamide, no heat, and resolved by 20% denaturing urea-PAGE.

These data suggest that band II corresponds to a Schiff base linkage between **C1** and 5′-product and thus provide strong evidence that a Schiff base had also formed between catalyst and substrate. Nevertheless, a Schiff base-linked substrate is not observed. Could it be that the lyase activity is sufficiently fast that the covalent intermediate between catalyst and substrate does not accumulate under pre-steady state, but disintegrates to the Schiff base-linked product instead?[37] We asked whether $NaCNBH_3$ or $NaBH_4$ could trap the Schiff base that putatively forms with **S1** and activates it for strand cleavage. These data are shown in Figure 6. Indeed, both $NaCNBH_3$ and $NaBH_4$ treatment following completion of strand cleavage gave a heat-stable product (band II) shown in lanes 2 and 5. When $NaBH_4$ was added at the outset of the reaction (lane 1), no new band arose. However when $NaCNBH_3$ was added similarly, a new product did arise (band IV). This band (i) was also stable to hot piperidine treatment (data not shown) and (ii) migrated more slowly than band II. This higher apparent molecular weight was consistent with trapping the ephemeral and heretofore undetected Schiff base linkage between **C1** and **S1**. The small quantity of covalently linked **C1**−**S1** that could be observed is consistent with its low level accumulation in the kinetic scheme.

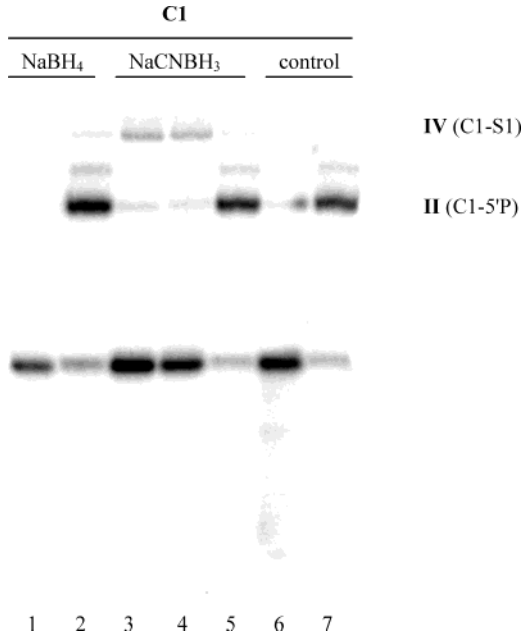


Figure 6. Lanes 1 and 3: reducing agent added 5 min after addition of catalyst to substrate and quenched after 30 min with 15 μL of formamide. Lane 4: reducing agent added after 5 min and quenched after 24 h with 15 μL of formamide. Lanes 2 and 5: reducing agent added after 24 h and quenched after 30 min with 15 μL of formamide. Lane 6: control. No reducing agent, quenched with 15 μL of formamide after 30 min. Lane 7: control. No reducing agent, quenched with 15 μL of formamide after 24 h. All reactions: 10 μL, 2.5 μM **C1**, 0.25 μM **S1**, 24−25 °C, in 500 mM NaCl, 50 mM Tris HCl pH 7.5, 1 mM EDTA. Reducing agent added for final concentration of 100 mM. Reactions quenched with 15 μL of formamide, no heat, and resolved by 20% denaturing urea-PAGE.

Notably, most of the substrate however did not react and no time-dependent accumulation of band IV was observed when $NaCNBH_3$ was allowed to reduce an incipient Schiff base linkage over a period of 24 h. This was ascribed either to $NaCNBH_3$ inactivation of **C1** via boronation of the amino functionalities,[38,39] reduction of the substrate, or both. A fainter band of intermediate mobility is also observed in this case and may represent a fortuitous and thus nonspecific interaction that is rendered irreversible upon reduction. Alternatively, this may be due to an intramolecular 1,4 Michael addition of a catalyst nucleophile, e.g. imidazole or allylamine, giving rise to a lariat form that then suffers subsequent reduction and results in an electrophoretically distinct species. Such 1,4 Michael addition to the resulting α,β-unsaturated enal/enimine has been observed in model systems.[40] It is also of note that with Lys-Trp-Lys and related peptides, multiple ancillary bands have been observed following $NaCNBH_3$ treatment.[41] We discount the significance of this product of intermediate electrophoretic mobility, as it represents but a small fraction of the total product.

To independently corroborate a Schiff base linkage to substrate, the effects of cyanide were also investigated. Cyanide, which quite effectively adds to imines to form cyanoamine adducts, is an effective inhibitor of enzymes that deliver Schiff base catalysis.[42] Nevertheless, in cases where cyanide is present in large excess over substrate, cyanide might inhibit activity by simply forming a cyanohydrin with substrate. To optimally address this concern, the effects of cyanide were investigated by using (a) a 3′-labeled substrate that results in a short 3′-labeled product and an unlabeled 5′-product; (b) a chase with both unlabeled 3′-product and a substrate analogue to effectively displace any labeled substrate that is noncovalently associated with the catalyst (see Figure 3); and (c) nondenaturing PAGE at 4 °C that gently resolves 3′-labeled product, 3′-labeled substrate, and any catalyst−substrate complexes that would include both chase-sensitive noncovalently associated species and chase-resistant, cyanoamine-linked species. These data are shown in Figure 7. Of primary importance are lanes 3 and 4, which demonstrate cyanide inhibition of lyase activity, and lanes 7 and 8, which demonstrate that the band corresponding to a catalyst−substrate interaction in the presence of cyanide was resistant to displacement following a 20 min chase. Reference bands corresponding to 3′-labeled product, 3′-labeled substrate, and a noncovalent interaction between catalyst and 3′-labeled substrate are given in lanes 9−11, respectively.

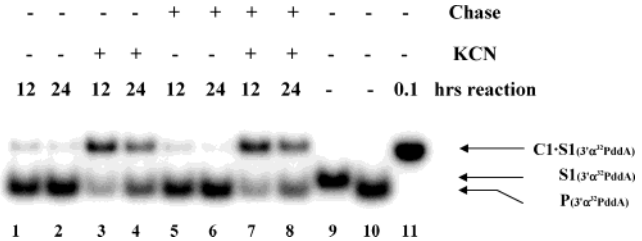


Figure 7. Lanes 1, 2, 5, 6: reaction of **C1** (2.5 μM) with trace 3′-labeled **S1** for 12 or 24 h in the absence of cyanide. Lanes 3, 4, 7, 8: reaction of **C1** (2.5 μM) with trace 3′-labeled **S1** for 12 or 24 h in the presence of 10 mM cyanide. Lanes 5, 6, 7, 8: chased for 20 min with a solution of 50 μM all-DNA substrate analogue and 50 μM 3′-product. Lane 9: 3′-labeled **S1** alone. Lane 10: 3′-labeled **S1** pretreated with piperidine. Lane 11: catalyst substrate complex preformed for 5−10 min prior to gel loading. Reaction proceeded at 22 °C in 500 mM NaCl, 50 mM Tris HCl pH 7.5, 1 mM EDTA. Reactions were chased for 20 min at the same temperature then placed at 0−4 °C or simply placed at 0−4 °C. All samples received 0.1 vol of 70% sucrose and loaded in a 20% native polyacrylamide gel run at 7 W for 2 h at 4 °C.

(36) This assumes that there are no slow steps to achieving catalytically competent conformations. See: Russell, R.; Herschlag, D. *J. Mol. Biol.* **2001**, *308*, 839−51 for a nice treatment of these effects.

(37) At this juncture in the formulation of the problem, we recognize the possibility of a Schiff base-independent cleavage mechanism that could result in syn elimination of the 3′-product, yielding a caged 5′-product that does not dissociate but is instead quantitatively dehydrated to a Schiff base linkage with the catalyst. The $NaCNBH_3$ trapping experiments would exclude this possibility.

Having demonstrated that lyase action is significantly faster than Schiff base formation, leading to low steady state accumulation of the Schiff base linkage to substrate and quantitative formation of a Schiff base-linked product, we wanted to address the chemical basis of a fast lyase activity. Contributing to a fast lyase activity would be the positioning of a general base near one of the 2′ protons. Could an imidazole be a likely general base at this position? This would certainly be consistent with the RNaseA activity of **C1**, where an imidazole is thought to play the role of a general base that deprotonates the 2′OH. In an attempt to implicate the imidazole as a general base in the lyase reaction and to retard this step so as to directly observe a covalent, heat-labile Schiff base linkage to substrate, the histaminyl groups were replaced with methylamino groups in the synthesis of **C2**. **C2** was then examined for its ability to form a Schiff base (Figure 8A, lanes 3/4) and effect elimination with or without exogenous 4MeIm (4-methylimidazole) (Figure 8B, lanes 1/2, 5/6).

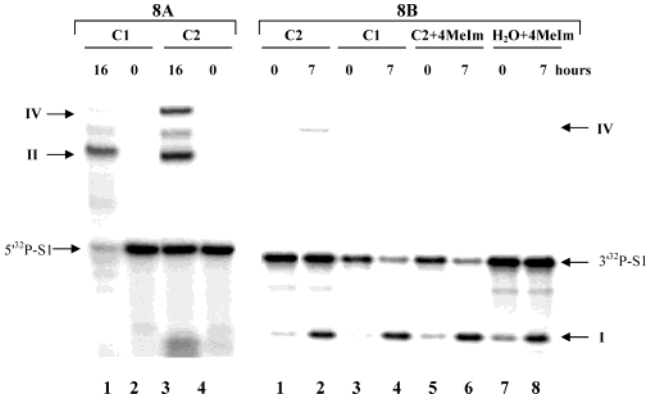


Figure 8. (A) Lanes 1 and 2: 5′-labeled **S1** with **C1**, 16, 0 h. Lanes 3 and 4: 5′-labeled **S1** with **C2** for 16 or 0 h. (B) Lanes 1 and 2: 0 and 7 h incubation of 3′-labeled **S1** with **C2**. Lanes 3 and 4: 0 and 7 h incubation of 3′-labeled **S1** with **C1**. Lanes 5 and 6 (200 mM 4MeIm): 0 and 7 h incubation of 3′-labeled **S1** with **C2**. Lanes 7 and 8: 0 and 7 h incubation of 3′-labeled **S1** absent **C2** (200 mM 4MeIm control). Lanes 1−4: 50 mM Tris HCl. Lanes 1−8: 50 mM $NaHPO_4$ pH 7.4 (MeIm-HPO_4 buffer prepared separately to ensure correct pH). All lanes also 500 mM NaCl, 1 mM EDTA. Samples quenched and resolved as in Figure 1.

With all imidazoles ablated, **C2** is catalytically impaired in terms of both Schiff base formation and lyase activity. Although at 2.5 μM **C2**, **S1** is completely saturated ($K_d \approx 30$ nM), the incapacity of **C2** to progress from a noncovalent base-paired association with substrate to a covalent Schiff base linkage is indicated by the large portion of free substrate that is dissociated by formamide treatment. The covalently linked species that are observed (Figure 8, lane 3) partition equally between adducts to 5′-product (band II) and to substrate **S1** (band IV), indicating that lyase activity ($k_{elim.}$) is also impaired on strands that are nevertheless capable of forming a Schiff base. Lyase activity was also examined with 3′-labeled **S1**, Figure 8B. In this case, a faint, retarded band was observed in lane 2 and corresponds to a link with 3′-labeled **S1** (gel in 8B not the same scale as in Figure 8A). The covalent linkage to **S1** now accumulates detectably and irrespectively of label (3′ or 5′), suggesting that lyase activity of **C2**, compared with that of **C1**, has been appreciably slowed.

With **C2**, only 22% strand scission (normalized against background) occurs in 7 h in contrast to the cleavage seen for **C1** (Figure 8B, lane 8). Exogenous addition of 200 mM 4MeIm restores **C2** cleavage to the level seen with **C1**. This is consistent with a similar experiment where 200 mM imidazole was used to rescue the HDV ribozyme.[43] In a control reaction, 200 mM 4MeIm gave only 13% cleavage (normalized). The 65% cleavage rescued by 4MeIm (i.e. **C2**+4MeIm) is greater than the sum of the cleavage yields observed for **C2** alone and 4MeIm alone.

Finally, the question of turnover was examined under both isothermal and variable-temperature conditions with excess substrate **S1** and limiting **C1**. Figure 9A shows multiple turnover when 0.25 μM **C1** is reacted with 1.25 μM 3′-labeled **S1**. In 17 h, approximately 50% cleavage is observed, corresponding to 2−3 turnover events (no background was observed in this time). Assuming that all the catalyst molecules are in an active conformation, this turnover translates into a k_{cat} of 2.4×10^{-3} min^{-1}, a value that is approximately 30−50% of that observed under single-turnover conditions.[44]

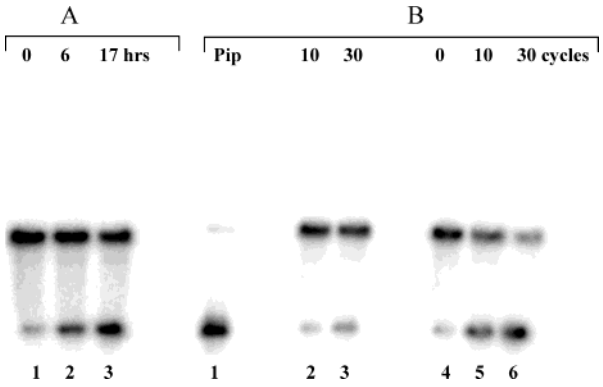


Figure 9. (A) Lanes 1−3 (multiple turnover): 0.25 μM **C1** and 1.25 μM 3′-labeled **S1** for 0, 6, and 17 h; 500 mM NaCl, 1 mM EDTA. (B) Lane 1: piperidine-treated control. Lanes 2 and 3: 10 and 30 cycles, respectively, without **C1**. Lanes 4−6: 0, 10, and 30 cycles with 0.25 μM **C1** and 2.5 μM 3′-labeled **S1**. Reaction conditions: 50 mM Tris HCl pH 7.5, 500 mM NaCl, 1 mM EDTA in all reactions. Samples quenched and resolved as in Figure 1.

As the Schiff base linkage to 5′-product was found to be surprisingly stable in a 7 M urea/polyacrylamide gel when cold-loaded, it is reasonable to suggest that hydrolysis of this linkage to yield 5′-product and regenerate free catalyst may be rate limiting. The heat lability of this linkage suggested that turnover numbers could be artificially boosted if the reaction were cycled between a low-temperature state that would permit sequence-specific association, Schiff base formation, and strand scission, and a high-temperature state that would promote unfolding and product release (hydrolysis of the Schiff base-linked product).

(38) (a) Miller, V. R.; Ryschkewitsch, G. E. *J. Am. Chem. Soc.* **1970**, *92*, 1558−62. (b) Mittakanti, M.; Morse, K. W. *Inorg. Chem.* **1990**, *29*, 554−6. (c) Bellon, L.; Workman, C. T.; Jarvis, T. C.; Wincott, F. E. *Bioconj. Chem.* **1997**, *8*, 204−12.
(39) $NaCNBH_3$ inhibited the RNaseA-like reaction catalyzed by **C1**, presumably due to cyanoboranation of the imidazoles, or amines, or both.
(40) (a) Vasseur, J.-J.; Rayner, B.; Imbach, J.-L.; Verma, S.; McCloskey, J. A.; Lee, M.; Chang, D.-K.; Lown, W. J. *J. Org Chem.* **1987**, *52*, 4994−8. (b) Mazumder, A.; Gerlt, J. A.; Absalon, M. J.; Stubbe, J.; Cunningham, R. P.; Withka, J.; Bolton, P. H. *Biochemistry* **1991**, *30*, 1119−26. (c) Zharkov, D. O.; Grollman, A. P. *Biochemistry* **1998**, *37*, 12384−94.
(41) (a) McCullough, A. K.; Sanchez, A.; Dodson, M. L.; Marapaka, P.; Taylor, J.-S.; Lloyd, R. S. *Biochemistry* **2001**, *40*, 561−8. (b) Kurtz, A. J.; Dodson, M. L.; Lloyd, R. S. *Biochemistry* **2002**, *41*, 7054−64.
(42) Cash, D. J.; Wilson, I. B. *J. Biol. Chem.* **1966**, *241*, 4290−2.
(43) Perrotta, A. T.; Shih, I. H.; Been, M. D. *Science* **1999**, *286*, 123−6.
(44) We have not addressed the issue of a burst-phase; as such the steady state k_{cat} would be further diminished.

By cycling the reaction temperature in a PCR thermocycler from 15 to 75 °C, 5 equivalent of substrate could be converted by 1 equiv of catalyst in 300 min. As a result, thermocycling increases the turnover number by a factor of 5 (Figure 9B).

Conclusions and Discussion

The DNAzyme we have investigated was initially selected from a family of DNA sequences capable of M^{2+}-independent ribophosphodiester hydrolysis, where both cationic amines and imidazoles would be positioned to mimic the active site of RNaseA. In the active site of RNaseA, the imidazole of histidine 119 plays the role of a base that deprotonates the 2′OH, whereas the amino group of lysine 41 is thought to play the role of electrostatic stabilization. **C1** contains 4 imidazoles and 6 cationic amines and presents us with the challenge of defining which of the 10 modifications are intimately involved in catalysis and which in folding. Using a 5′-labeled substrate containing an abasic site, it was evident that the catalyst became covalently linked to the 5′-product in the course of the reaction at roughly the same rate as strand cleavage was observed with a 3′-labeled substrate. *A priori*, this profile is wholly consistent with a Schiff base-dependent APE activity. This key observation prompted a more thorough characterization of M^{2+}-independent strand scission of an abasic site. The data leave little doubt as to the nature of the 5′-labeled product appearing in band II, which represents a Schiff base linkage between catalyst and product that is rendered stable to hot piperidine by borohydride treatment. Borohydride trapping at early time points of the reaction resulted in the appearance of another band of higher apparent molecular weight and thus allowed for inference of a putative Schiff base linkage between substrate and catalyst. This linkage does not accumulate due to a more rapid lyase reaction resulting in elimination and strand cleavage.

A concern that must be entertained as we err on the side of caution is to consider the remote possibility that Schiff base catalysis was not operative and that the $NaCNBH_3$ trapping of **S1** on **C1** was simply a fortuitous event favored by a thermodynamically irreversible reduction as others have cautioned in their own work, and even exploited in the study of a non-APE.[45] Indeed, Grollman has specifically questioned the value of borohydride trapping, which has long been considered a hallmark of Schiff base catalysis.[46] Without Schiff base catalysis, 5′- and 3′-labeled substrates would have resulted in faster migrating products under denaturing PAGE. Instead, only the 3′-labeled substrate gave a single, faster moving product, whereas the 5′-labeled substrate gave rise to a product of higher apparent molecular weight that coincided with an identical product observed when the catalyst was labeled instead of substrate.

The only way to reconcile the argument that Schiff base catalysis is inoperative with the observation of a linkage between catalyst and 5′-product is to invoke the argument of product stabilization where Schiff base linkage to product occurs quantitatively and without detectable product diffusion *after* strand scission. For such a scenario, we must then stipulate that the 5′-product be tightly held within an "active site" and that Schiff base formation occurs in a concerted fashion following strand scission along a reaction path that does not permit the observation of any diffusible 5′-labeled product corresponding to a 7-mer product. We consider such a scenario, while within the realm of possibility, to be rather unlikely.

To further substantiate that the catalyst formed a Schiff base with substrate *prior* to strand scission, we applied an orthogonal test that Grollman has proposed, namely, that cyanide inhibition should also be examined as a further indication of Schiff base formation.[47] Cyanide forms reversible covalent adducts with imines and is thus an inhibitor of enzymes that operate via covalent Schiff base catalysis. Indeed, cyanide was found to inhibit the AP-lyase activity when monitored with 3′-labeled substrate under denaturing conditions (data not shown). When monitored by denaturing PAGE, inhibition with cyanide in excess over substrate, could be attributed to either direct effects (cyanoamine formation) or indirect effects (cyanohydrin formation).

The use of native PAGE and a chase reaction permitted differentiation of these two scenarios. In the case of cyanohydrin inhibition, the association of catalyst with radiolabeled cyanohydrin adduct would be noncovalent and consequently could be gently displaced with a chase using excess unlabeled 3′-product and a substrate analog. In the case of direct inhibition (cyanoamine), the cyanide-mediated covalent linkage would not be susceptible to displacement in the chase reaction. Lyase activity, and any inhibition thereof by cyanide, would still be observed by virtue of the 3′-labeling, which would give rise to a 3′-labeled product that would dissociate from **C1** under nondenaturing conditions. This was indeed found to be the case; cyanide not only inhibits product formation but also reacts with the Schiff base linkage between **C1** and **S1** to give rise to a chase-resistant species that can be resolved in the native gel. When taken with the rest of the data, these findings quite conclusively demonstrate that Schiff base formation precedes product formation and is thus the slow step of the reaction.

What then is a reasonable value for $k_{elim.}$? Indeed, this value remained difficult to address in this initial characterization where Schiff base formation seemed to be rate-limiting compared to elimination. For the Schiff base linkage to substrate not to accumulate detectably, $k_{elim.}$ must be at least 10 times greater than the rate-limiting step, $k_{Schiff\ base}$, or on the order of 0.06 min^{-1}. Although the value of $k_{elim.}$ could be much higher, this value represents a reasonable estimate that is in keeping with k_{cat} of the M^{2+}-independent RNA cleavage observed for **C1**. This suggests the potential for overlapping conformations with a redundancy of functionality. A complete structure−function analysis will identify the exact catalytic imidazole(s) and amines along with the stereochemical outcome of an elimination that should result in a trans olefinic aldehyde if this catalyst operates via the same mechanism as other APEs and their mimics, as shown by Gerlt and co-workers and Lloyd and co-workers.[48]

Not to be underestimated is the role of the imidazole to enzyme catalysis. This case is no exception: when the imidazole was ablated, subdued activity for both Schiff base formation

(45) (a) Mazumder, A.; Neamati, N.; Pilon, A. A.; Sunder, S.; Pommier, Y. *J. Biol. Chem*. **1996**, *271*, 27330−8. (b) McCullough, A. K.; Sanchez, A.; Dodson, M. L.; Marapaka, P.; Taylor, J.-S.; Lloyd, R. S. *Biochemistry* **2001**, *40*, 561−8.

(46) Zharkov, D. O.; Grollman, A. P. *Biochemistry* **1998**, *37*, 12384−94.

(47) (a) Dodson, M. L.; Schrock, R. D.; Lloyd, R. S. *Biochemistry* **1993**, *32*, 8284−90. (b) Dodson, M. L.; Michaels, M. L.; Lloyd, R. S. *J. Biol. Chem*. **1994**, *269*, 32709−12.

(48) (a) McCullough, A. K.; Sanchez, A.; Dodson, M. L.; Marapaka, P.; Taylor, J.-S.; Lloyd, R. S. *Biochemistry* **2001**, *40*, 561−8. (b) Kurtz, A. J.; Dodson, M. L.; Lloyd, R. S. *Biochemistry* **2002**, *41*, 7054−64.

and strand scission was observed, suggesting that this functionality may be involved both in general acid/base catalysis that might facilitate Schiff base formation as well as general base catalysis for strand scission. We cautiously suggest that rescue by 4MeIm is certainly consistent with the role of an imidazole base on **C1** participating to enhance elimination, particularly in light of the fact that **C1** exhibits RNaseA-like activity.

Is the primary alkyl amine directly involved in the Schiff base linkage as chemical intuition would suggest? We contend that the Schiff base linkage is likely to involve the primary alkyl amine since no reaction was observed in the absence of both modifications but was observed in the absence of only the imidazole. Nevertheless, we cannot absolutely exclude the possibility that the modified bases might act indirectly to properly fold a catalytic domain and perturb the activities of the exocyclic amines on unmodified DNA components via new H-bonding motifs, electrostatic interactions, and even hydrophobic effects to enable the exocyclic amines on any of these bases to engage in Schiff base formation.[49] If the observed activity is in fact due to *unmodified* bases contained within new folding motifs of potentially altered dielectric constants involving the modified bases, such structures should prove quite interesting as well.

So far, emphasis has been placed on characterizing the first steps of this reaction under single-turnover conditions to make a case for Schiff base formation. In the development and characterization of catalysts, the feature of turnover is integral. In this case, turnover, although modest, was observed. The rate of turnover was diminished compared to that measured under single-turnover conditions and is most likely due to slow product release relating to hydrolysis of a particularly stable Schiff base linkage between catalyst and product. To demonstrate this, we sought to increase turnover rates through the use of thermocycling. The use of thermocycling is another salient point of this work; it demonstrates the robust nature of modified DNAzymes and to the best of our knowledge is only the second report of thermocycling of a DNAzyme.[50]

Beyond demonstrating the robust nature of modified DNAzymes in variable-temperature catalysis, thermocycling may find application in kinetically complicated reactions where thermocycling can be used to deliver optimal temperatures for varying steps of a multistep reaction. Thermocycling may also be used in a qualitative fashion to examine kinetic schemes where slow product release, such as Schiff base hydrolysis, proves rate limiting at low temperature, but which can be enhanced by heating. In a thermocycled reaction, kinetics are not necessarily simplified. However a judicious choice of temperature regimes may be used to change the rate-limiting step over the course of the reaction such that the catalyst can achieve several conformational states during the course of the reaction. These altered states may be advantageous to substrate recognition, chemical catalysis, and product release. This indeed is the basis for the exponential kinetics observed with semiconservative DNA replication in a thermocycled PCR. In such a scheme, the rate-limiting step will still be determined either by the activation energy of the most energetically demanding step or by the heat capacity of the reaction vessel that governs the rate of temperature change.

One might ask, what might be the utility of a thermocycled DNAzyme APE? Recently, a selection intended to generate a DNAzyme with β-glycosidase activity adventitiously resulted in a M^{2+}-dependent depurinase activity that did not appear to react further with the abasic site to deliver strand scission.[51] Tandem action by a DNAzyme depurinase and a DNAzyme APE under cycling conditions would enable an efficient and site-specific depurination of chromosomal DNA followed by lyase-mediated cleavage of the same. These systems may find utility for DNA manipulations in cases where no restriction enzyme exists and where oxidative scission might be disadvantageous.

It is noteworthy that this APE activity, while neither enhanced nor inhibited by either Mg^{2+} or Ca^{2+} (data not shown), proceeds in the absence of either cation. Whereas M^{2+}-independent RNase activity has been observed in *un*modified nucleic acids, both natural[52] and selected,[53] this AP-lyase activity has no precedent in the "nucleic acid world". The lyase activity presented herein is reminiscent of class-I aldolases that accomplish carbon−carbon bond formation and cleavage by similar covalent intermediates. Nature uses both M^{2+}-dependent and -independent strategies. For example, class II aldolases and certain APEs use divalent metal cations instead of a primary amine. In the absence of a divalent metal cation, Schiff base catalysis provides an alternative solution to catalyzing energetically demanding reactions. In the absence of both an alkylamine and a divalent metal cation, it is challenging to imagine how a catalyst would evolve to accomplish an E1cB/β elimination as observed here.[54] Selections dedicated to APE discovery should test whether the nucleobase nitrogens in unmodified catalysts can be capable of Schiff base catalysis.

This DNAzyme exhibits both RNaseA and APE activities, consistent with the action of a general base that effects 2′OH-deprotonation in the former and 2′H-deprotonation in the latter. As such, this DNAzyme is catalytically promiscuous. Catalytic promiscuity has been the subject of recent reviews and is not unique to either nucleic acids or proteins.[55] Examples include the Tetrahymena ribozyme that catalyzes aminoacylation reactions,[56a] the hammerhead ribozyme that catalyzes the deglycosylation of 2′-mercaptocytidine,[56b] a combinatorially selected calcium-dependent metalloribozyme that exhibits both decapping and pyrophosphatase activity,[56c] the catalytic antibody 38C2 that uses a lysine in Schiff base catalysis for aldol chemistry and the same as a base to catalyze a Kemp

(49) Kirby, A. J.; Marriott, R. E. *J. Am. Chem. Soc.* **1995**, *117*, 833−4.
(50) Cairns, M. J.; King, A.; Sun, L.-Q. *Nucl. Acids Res.* **2000**, *28e9*, i−vi.
(51) Sheppard, T. L.; Ordoukhanian, P.; Joyce, G. F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7802−7.
(52) (a) Wadkins, T. S.; Shih, I.-H.; Perrotta, A. T.; Been, M. D. *J. Mol. Biol.* **2001**, *305*, 1045−55. (b) Perrotta, A. T.; Shih, I. H.; Been, M. D. *Science* **1999**, *286*, 123−6. (c) O'Rear, J. L.; Wang, S.; Feig, A. L.; Beigelman, L.; Uhlenbeck, O. C.; Herschlag, D. *RNA* **2001**, *7*, 537−45. (d) Murray, J. B.; Seyhan, A. A.; Walter, N. G.; Burke, J. M.; Scott, W. G. *Chem. Biol.* **1998**, *5*, 587−95.
(53) (a) Jayasena, V. K.; Gold, L. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10612−7. (b) Geyer, C. R.; Sen, D. *Chem. Biol.* **1997**, *4*, 579−93. (c) Faulhammer, D.; Famulok, M. *J. Mol. Biol.* **1997**, *269*, 188−202.
(54) Koch, T.; Reymond, J.-L.; Lerner, R. A. *J. Am. Chem. Soc.* **1995**, *117*, 9383−7, provides an interesting exception where neither a divalent metal cation nor a Schiff base is observed.
(55) (a) O'Brien, P. J.; Herschlag, D. *Chem. Biol.* **1999**, *6*, R91−105. (b) Copley, S. D. *Curr. Opin. Chem. Biol.* **2003**, *7*, 265−72.
(56) (a) Piccirilli, J. A.; McConnell, T. S.; Zaug, A. J.; Noller, H. F.; Cech, T. R. *Science* **1992**, *256*, 1420−4. (b) Hamm, M. L.; Schwans, J. P.; Piccirilli, J. A. *J. Am. Chem. Soc.* **2000**, *122*, 4223−4. (c) Huang, F. Q.; Yarus, M. *Biochemistry* **1997**, *36*, 14107−19. (d) James, L. C.; Tawfik, D. S. *Protein Sci.* **2001**, *10*, 2600−7. (e) Backes, A. C.; Hotta, K.; Hilvert, D. *Helv. Chim. Acta* **2003**, *86*, 1167−74. (f) Branneby, C.; Carlqvist, P.; Magnusson, A.; Hult, K.; Brink, T.; Berglund, P. *J. Am. Chem. Soc.* **2003**, *125*, 874−5.

elemination,[56d] the antibody 21D8 that catalyzes decarboxylation and ester hydrolysis,[56e] and a serine hydrolase from C. *antarctica* that catalyzes aldol condensation.[56f]

What is unique to this work, at least to date, is the demonstration of Schiff base catalysis by a modified DNAzyme. The findings herein provide yet another example of how synthesis can bridge the gap between nucleic acids and proteins and demonstrate how a synthetically appended primary amine can enhance the catalytic repertoire of nucleic acids to now include covalent Schiff base catalysis. It is not difficult to imagine a selection in the near future that will describe a DNAzyme capable of class-I-like aldolase activity, a key step in metabolism. In a prebiotic world where nucleic acids would be required to accomplish metabolism and maintain genetic inheritance, genetic material endowed with added functionality is an attractive idea. Experimental evidence for the acquisition of such modification under more prebiotic conditions had been reported and provided inspiration for this work.[57]

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Supporting Information Available: Information for protocols and extra data are available on the web at http://pubs.acs.org.

JA037625S

(57) Robertson, M. P.; Miller, S. L. *Science* **1995**, *268*, 702−5.