Enhanced Ligation of DNA with a Synthetic Effector Molecule

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DNA ligases catalyze phosphodiester bond formation between 5′-phosphate and 3′-hydroxyl ends of DNA fragments, with the pyrophosphate bonds of ATP or NAD cofactors as a source of energy. However, for some therapeutic approaches using synthetic circular or modified oligonucleotides, *in vivo* experiments will require large quantities of compounds which cannot be obtained enzymatically. Chemical solutions to the ligation of nucleic acids have appeared, using carbodiimides or cyanogen bromide and its derivatives for phosphate end activation in an aqueous medium, in a double[1,2] or triple helix[3] template. However, yields are low, and extended reaction times and a large excess of activating agent are required, both of which may be harmful to the oligonucleotide.

As a further step toward the long-term goal of designing enzyme mimics,[4,5] we report here that *a spermine–histamine conjugate acts as a ligase substitute in enhancing the rate and yield of cyanoimidazole (cofactor)-promoted ligation of DNA.* Indeed, we previously showed that polyamines crawl along the DNA minor groove floor with little sequence selectivity.[6] Thus spermine is an ideal vehicle to carry the potential acid/base catalyst imidazole[7] in the neighborhood of the nick, where phosphate and hydroxyl ends are maintained close to each other by the intact strand.[8]

L-Ornithine was successively biscyanoethylated, and nitrile residues were reduced with hydrogen and amines protected as *tert*-butyl carbamate esters.[5] The resulting tetrakis(*tert*-butoxycarbonyl)spermine-5-carboxylic acid was converted to its *N*-hydroxysuccinimide ester with DCC and then condensed with histamine and subsequently deprotected in TFA, giving spermine-5-(*N*-ethylimidazole)carboxamide (SperIm, Figure 1) in 70% overall yield.[9]

A 30-base oligonucleotide template (GCTACGCCAACAAAAAGGAAGAGTATAGCG, 30 mer), as well as two oligonucleotides (CGCTATACTCTTCCTTT, 17mer, and TTCTTGGCGTAGC, 13mer) complementary to this 30mer, was synthesized chemically and purified by polyacrylamide gel electrophoresis. This latter oligonucleotide was 5′-phosphorylated with polynucleotide kinase and ATP or (γ-^{32}P)ATP (P-13mer). Equimolar amounts of the three oligonucleotides were mixed to give the nicked duplex (30mer:17mer P-13mer) (1 μM) of which the 5′-phosphate belonging to the reaction site was radioactively labeled. Ligation was followed by monitoring the ratio of radioactive starting 13mer to newly formed 30mer after denaturating 20% polyacrylamide gel electrophoresis (experimental conditions in figure legends).

In a first set of experiments performed near room temperature (30 °C, Figure 2, lanes 1–6), cyanoimidazole[10] (ImCN) at 10 mM proved to be a poor ligating agent[11] (15% after 18 h, lane 3), even in the presence of Mg^{2+} (16%, lane 4).[12] However, addition of SperIm (0.1 mM) to the reaction mixture resulted in

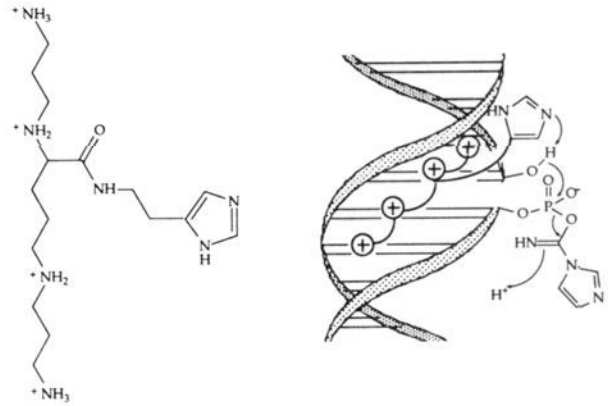

Figure 1. (Left) structure of SperIm; (right) ligation pathway of this effector molecule with ImCN as cofactor.

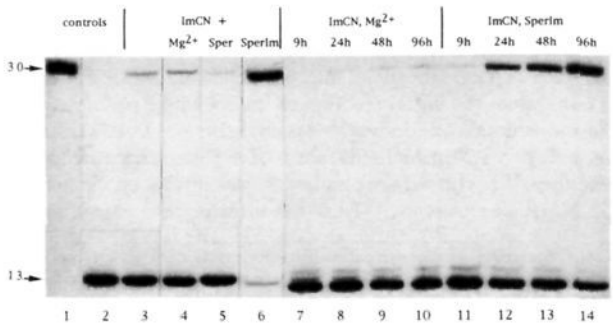

Figure 2. Autoradiograms of 20% polyacrylamide denaturing gels. Synthesis of a radioactive 30mer (30→) by ligation of a 5′-^{32}P-phosphorylated 13mer (13→) with a 17mer on the complementary strand. Reaction mixtures contained 1 μM concentration of the nicked duplex (30mer:17mer P-13mer) in a total volume of 10 μL. Lane 1: ligation product of T4 DNA ligase (1 U). Lane 2: duplex alone. Lanes 3–6: reaction mixtures initially containing 10 mM ImCN and 10 mM MES buffer pH 5.5. After incubation for 18 h at 30 °C, the samples were dried under vacuum and dissolved in gel loading buffer. Lane 4: 10 mM $MgCl_2$. Lane 5: 100 μM spermine. Lane 6: 100 μM SperIm. Lanes 7–14: samples containing 5 mM ImCN in 10 mM MES buffer pH 6.0 and either 10 mM $MgCl_2$ (lanes 7–10) or 50 μM SperIm (lanes 11–14). They were incubated at 4 °C for 9, 24, 48, and 96 h.

(1) Naylor, R.; Gilham, P. T. *Biochemistry* **1966**, *5*, 2722–2728.
(2) (a) Orgel, L. E.; Lohrman, R. *Acc. Chem. Res.* **1974**, *7*, 368–377. (b) Shabarova, Z. A.; Prokofiev, M. A. *FEBS Lett.* **1970**, *11*, 237–240. (c) Shabarova, Z. A.; Dolinnaya, N. G.; Drusta, V. L.; Melnikova, N. P.; Purmal, A. A. *Nucleic Acids Res.* **1981**, *9*, 5747–5761. (d) Kanaya, E.; Yanagawa, H. *Biochemistry* **1986**, *25*, 7423–7430. (e) Dolinnaya, N. G.; Sokolova, N. I.; Gryaznova, O. I.; Shabarova, Z. A. *Nucleic Acids Res.* **1988**, *16*, 3721–3738. (f) Ashley, G. W.; Kushlan, D. M. *Biochemistry* **1991**, *30*, 2927–2933. (g) Dolinnaya, N. G.; Sokolova, N. I.; Ashirbekova, D. T.; Shabarova, Z. A. *Nucleic Acids Res.* **1991**, *19*, 3067–3072. (h) Dolinnaya, N. G.; Tsytovich, A. V.; Sergeev, V. N.; Orestskaya, T. S.; Shabarova, Z. A. *Nucleic Acids Res.* **1991**, *19*, 3073–3080.
(3) (a) Luebke, K. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 8733–8735. (b) Luebke, K. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1991**, *113*, 7447–7448. (c) Kool, E. T. *J. Am. Chem. Soc.* **1991**, *113*, 6265–6266. (d) Luebke, K. J.; Dervan, P. B. *Nucleic Acids Res.* **1992**, *20*, 3005–3009.
(4) (a) Behr, J. P.; Lehn, J. M. *J. Chem. Soc., Chem. Commun.* **1978**, 143–146. (b) Lehn, J. M.; Sirlin, C. *Ibid.* **1978**, 949–951. (c) Breslow, R.; Doherty, J.; Guillot, G.; Lipsey, C. *J. Am. Chem. Soc.* **1978**, *100*, 3227–3229. (d) Chao, Y.; Weisman, G. R.; Sogah, G. D. Y.; Cram, D. J. *J. Am. Chem. Soc.* **1979**, *101*, 4948–4958. (e) Rebek, J.; Marshall, L.; Wolak, R.; Parris, K.; Killoran, M.; Askev, B.; Nemeth, D.; Islam, N. *J. Am. Chem. Soc.* **1985**, *107*, 7476–7481. (f) Jubian, V.; Dixon, R. P.; Hamilton, A. D. *J. Am. Chem. Soc.* **1992**, *114*, 1120–1121.
(5) Behr, J. P. *J. Chem. Soc., Chem. Commun.* **1989**, 101–103.
(6) Schmid, N.; Behr, J. P. *Biochemistry* **1991**, *30*, 4357–4361.
(7) Breslow, R. *Acc. Chem. Res.* **1991**, *24*, 317–324.
(8) (a) Ayamami, J.; Coll, M.; Van Der Marel, G. A.; Van Der Boom, J. H.; Wang, A. H.-J.; Rich, A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 2526–2530. (b) Snowden-Ifft, E. A.; Wemmer, D. E. *Biochemistry* **1990**, *29*, 6017–6025.

(9) Tetrakis(*tert*-butoxycarbonyl)spermine-5-(*N*-ethylimidazole)carboxamide: MS FAB *m/z* 740.4 (MH^+). Anal. Calcd for $C_{36}H_{65}N_7O_9$ (Found): C, 58.4 (58.2); H, 8.8 (8.7); N, 13.2 (13.0). SperIm was obtained as a hygroscopic hydrotrifluoroacetate salt: ^{1}H NMR (200 MHz, CD_3OD), ppm 1.6–2.2 (8H, m), 2.8–3.2 (10H, m), 3.5 (2H, t), 3.9 (1H, d J = 6 Hz), 7.4 (1H, d, J = 1 Hz), 8.8 (1H, d, J = 1 Hz); MS FAB *m/z* 340.2 (MH^+).
(10) *N*-Cyanoimidazole was prepared as described: Giesemann, H. *J. Prakt. Chem.* **1955**, *1*, 345–348.
(11) The reaction product is identified by comigration with the T4-DNA ligase product, lane 1.
(12) Magnesium as well as other divalent cations improves the yield of ligation.[2f-h]

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a substantial increase of ligated oligonucleotide (80%, lane 6).[13] Such an increase cannot be accounted for by the stabilizing effect of the spermine moiety on dangling reacting oligonucleotide ends (spermine actually decreases the yield to 10%; lane 5), nor is it due solely to the presence of imidazole (data not shown). Thus SperIm is unique in combining a DNA binding moiety with a putative catalyst.[14]

Next, for convenience, we followed the time course of the reaction at 4 °C, where ImCN has extended lifetime ($t_{1/2}$ = 72 h in 10 mM MES pH 6, as compared to 4 h at room temperature) and where the duplex is fully formed (T_m = 40 °C). In the presence of SperIm (50 μM) the reaction progresses smoothly (Figure 2, lanes 11–14), whereas ImCN (5 mM)/Mg^{2+} (10 mM) shows ligation that is hardly detectable (lanes 7–10). This experiment was repeated several times in order to approximate the kinetic enhancement factor. Figure 3 shows a plot of % ligation vs time over a 4-day period: after a ca. 9-h lag time,[15] the SperIm-enhanced reaction proceeds about 50-fold faster than the simple chemical ligation reaction.

The mechanism of ImCN or BrCN/buffer activation of phosphoesters is still unclear;[2d,h,16] iminoimidazolide phosphoesters and/or phosphoimidazolides may be intermediates. In any case, in our system the initial reaction of ImCN with SperIm to give the iminodiimidazole derivative[17] is unlikely since ImCN is stable for 1 day in 200 mM imidazole buffer,[2d] and the absence of reaction of SperIm with excess ImCN was further confirmed by ^{1}H NMR spectroscopy. Thus the effector molecule SperIm probably acts as a general acid/base catalyst to favor reaction of the ribose 3′-hydroxyl- and ImCN-activated 5′-phosphate termini (Figure 1).[18]

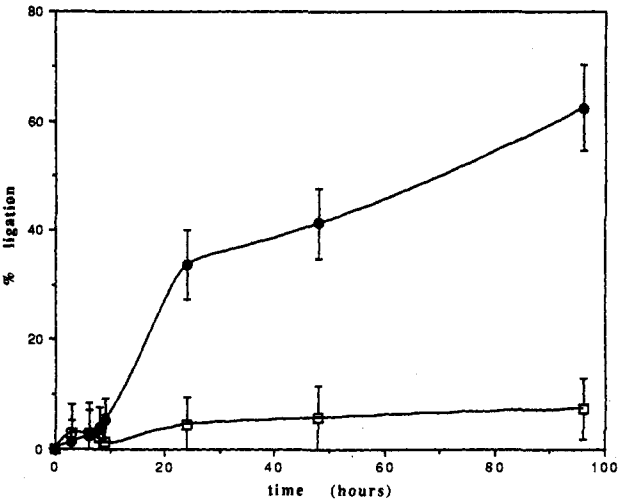

Figure 3. Plot of ligation yield vs time in the presence of 50 μM SperIm (•) or 10 mM $MgCl_2$ (□). Reaction mixtures contained 1 μM concentration of the nicked duplex (30mer:17mer P-13mer), 5 mM ImCN, and 10 mM MES buffer pH 6.0 in a total volume of 10 μL. The yield of ligation was estimated by Bioimager (BAS 2000, Fuji) analysis of a 20% polyacrylamide denaturing gel.

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(13) Moreover, it is known that such ligation reactions involving secondary hydroxyl attack on an activated 5′-phosphate proceed with lower yield than primary hydroxyl reactions on a 3′-phosphate.[2e,g]

(14) Imidazole buffer catalysis has been observed in oligoribonucleotide ligation[2d] and hydrolysis[7] reactions.

(15) Such low-temperature lag time has been observed previously.[2d]

(16) Ferris, J. P.; Huang, C.-H.; Hagan, W. J., Jr. *Nucleosides Nucleotides* **1989**, *8*, 407–414.

(17) Iminodiimidazole and carbonyldiimidazole may lead to reactive phosphoimidazolides.[2d,16]

(18) This mechanism remains to be demonstrated; yet the observed lag time and transient appearance of a slightly slower moving P-13mer component in the absence of catalyst (Figure 2) also favor a two-step mechanism of ImCN-mediated phosphate activation followed by a slower ligation reaction.