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Selective Recognition of Unnatural Imidazopyridopyrimidine:Naphthyridine Base Pairs Consisting of Four Hydrogen Bonds by the Klenow Fragment

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Selective recognition of complementary base pairs during DNA replication by DNA polymerase is a fundamental biological process in transmitting genetic information. Since this ubiquitous event occurs in all living matter using only two sets of base pairs consisting of adenine (A):thymine (T) and guanine (G):cytosine (C), the creation of alternative new base pair(s) other than the Watson−Crick base pairs, which could replicate selectively, would be a potentially useful contribution to the field. Starting with the pioneering work of Benner's group,[1] the development of such base pairs has been intensely investigated to expand the genetic code and explore synthetic biology.[2] Throughout these aforementioned investigations, it has been suggested that shape complementarity of the purine:pyrimidine pair and hydrophobic (stacking) interactions between the nucleobases as well as the complementarity of the hydrogen bonds (H-bonds) are critical for the selective recognition of DNA polymerases.

We have been working on a project to develop new base pairs consisting of four H-bonds.[3] Accordingly, we designed imidazopyridopyrimidine (Im):naphthyridine (Na) base pairs and found that the DNA duplexes with ImO^N:NaN^O and ImN^O:NaO^N pairs were highly thermally stabilized (+8−9 °C per pair) resulting from (1) four noncanonical H-bonds, (2) stacking ability, and (3) shape complementarity of the Im:Na pair (Figure 1).[4] These successful results prompted us to investigate how these thermally stable base pairs are recognized by DNA polymerases. In this communication, we report the results of kinetic studies of Im:Na base pair recognition by the Klenow fragment (KF).

We began the proposed study by preparing the corresponding nucleoside 5′-triphosphates, ImO^NTP, ImN^OTP, NaN^OTP, and NaO^NTP (Supporting Information, Schemes S1 and S2), and then we examined single nucleotide insertion into a template−primer duplex (Figure S1) by KF. As can be seen in Figure 2, NaN^OTP was incorporated against ImO^N in the template to afford a 21-mer sequence (Figure 2A, lane 7)[5] while other dNTPs were not incorporated at all (lanes 2−6). When NaN^O was introduced in the template, dATP as well as ImO^NTP was incorporated as the complementary 5′-triphosphate (Figure 2B, lanes 8 and 12). When the same reactions were carried out in the ImN^O:NaO^N pair (Figure S2), a higher selectivity was observed.

To understand these observations quantitatively, we determined the kinetic parameters (K_m = the Michaelis constant, V_{max} = the maximum rate of the enzyme reaction, and V_{max}/K_m = the insertion efficiency) of every 5′-triphosphate at various concentrations (Table 1). The quantitative analyses revealed that KF incorporated NaN^OTP preferentially against ImO^N in the template, and the efficiency was 100−1000-fold higher than other dNTPs (V_{max}/K_m; 8.5×10^6 vs 2.3×10^4−5.1×10^3). Although the efficiency of ImO^NTP incorporation against NaN^O was slightly higher than that of NaN^OTP against ImO^N (V_{max}/K_m; 2.5×10^7 vs 8.5×10^6), incorporation of dATP also showed the same efficiency (V_{max}/K_m; 2.9×10^7 vs 2.5×10^7). For the ImN^O:NaO^N pair, either ImN^OTP or NaO^NTP was

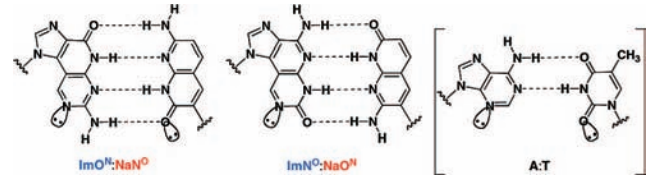

Figure 1. Structures of base pairs consisting of four H-bonds.

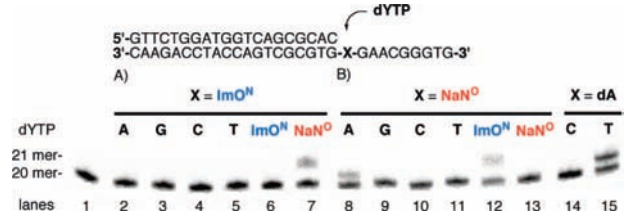

Figure 2. Single nucleotide insertion by Klenow fragment (selectivity toward natural dNTPs). (A) Incorporation of dYTP against ImO^N in template. (B) Incorporation of dYTP against NaN^O in template. In lanes 14 and 15, the results of matched and mismatched pairs of natural substrates were shown. Experimental details are described in the Supporting Information.

incorporated selectively against NaO^N and ImN^O, respectively, in the templates although the efficiencies were ∼1 order of magnitude lower than those of ImO^NTP and NaN^OTP (V_{max}/K_m; 8.5×10^6 vs 2.3×10^5 and 2.5×10^7 vs 3.6×10^6, respectively).

A careful consideration of these results indicates first that noncanonical base pairs consisting of four H-bonds were, interestingly, recognized preferentially by KF as complementary bases. Although one can imagine, for example, a T:ImO^N pair with three H-bonds (Figure S3), TTP incorporation was approximately a few thousand-fold less than NaN^OTP, and enzymatic recognition of the pair with four H-bonds by KF was thought to act advantageously. On the other hand, the efficiency of dATP incorporation against NaN^O was almost equal to that of ImO^NTP despite the fact that only two H-bonds can be expected in the A:NaN^O pair (Figure S3). This result suggests that NaN^O in the template would be recognized as a ring-expanded T analogue. For the ImN^O:NaO^N pair, the selectivities against natural dNTPs were higher than those of the ImO^N:NaN^O pair, although the efficiencies of the NaO^NTP and ImN^OTP incorporation were somewhat lower. These results can be attributed to the H-bonding pattern of ImN^O:NaO^N pair. Thus, it has been suggested that interaction of the N3 of the purine base and the O2 of the pyrimidine base as proton acceptors located in the minor groove with the DNA polymerase is critical for dNTP incorporation (see A:T pair in Figure 1).[6] In the case of the ImO^N:NaN^O pair, a similar interaction is expected as depicted in Figure 1, while the proton acceptor corresponding to the O2 of the pyrimidine base is missing in the ImN^O:NaO^N pair. Therefore, this unusual H-bonding pattern is thought to exhibit higher selectivity, albeit a lower efficiency for the ImN^O:NaO^N pair relative to the

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Table 1. Steady-State Kinetics Data of the Single Nucleotide Insertion by Klenow Frangemt[a]

X	dYTP	K_m (μM)	V_{max} (%·min^{-1})	V_{max}/K_m (%·min^{-1}·M^{-1})
ImO^N	dATP	19 ± 10	0.43 ± 0.033	2.3 x 10^4
	dGTP	34 ± 2.6	0.18 ± 0.007	5.3 x 10^3
	dCTP	51 ± 13	0.44 ± 0.044	8.6 x 10^3
	TTP	54 ± 18	0.28 ± 0.034	5.1 x 10^3
	ImO^NTP	–	–	n. d.[b]
	NaN^OTP	2.6 ± 0.49	22 ± 0.96	8.5 x 10^6
NaN^O	dATP	0.69 ± 0.22	20 ± 3.4	2.9 x 10^7
	dGTP	17 ± 1.8	9.4 ± 1.5	5.5 x 10^5
	dCTP	25 ± 5.5	0.12 ± 0.010	4.8 x 10^3
	TTP	19 ± 0.14	0.11 ± 0.015	5.8 x 10^3
	ImO^NTP	0.83 ± 0.23	21 ± 5.3	2.5 x 10^7
	NaN^OTP	14 ± 3.8	0.32 ± 0.019	2.3 x 10^4
ImN^O	dATP	120 ± 6.4	0.65 ± 0.047	5.4 x 10^3
	dGTP	43 ± 10	0.095 ± 0.007	2.2 x 10^3
	dCTP	–	–	n. d.[b]
	TTP	16 ± 4.6	0.12 ± 0.019	7.5 x 10^3
	ImN^OTP	–	–	n. d.[b]
	NaO^NTP	57 ± 4.1	13 ± 1.5	2.3 x 10^5
NaO^N	dATP	25 ± 3.1	16 ± 2.1	6.4 x 10^5
	dGTP	33 ± 7.7	0.15 ± 0.027	4.5 x 10^3
	dCTP	71 ± 31	0.25 ± 0.051	3.5 x 10^3
	TTP	20 ± 2.5	0.13 ± 0.012	6.5 x 10^3
	ImN^OTP	8.3 ± 2.5	27 ± 4.7	3.6 x 10^6
	NaO^NTP	29 ± 2.9	2.4 ± 0.56	8.3 x 10^4
T	dATP	0.43 ± 0.01	26 ± 1.4	6.0 x 10^7
A	TTP	0.30 ± 0.05	27 ± 3.2	9.0 x 10^7

[a] Experimantal details and fidelity against corrected base pair (Table S1) were presented in the Supporting Information. [b] The reaction was too insufficient to determine the kinetic parameters.

ImO^N:NaN^O pair. Additionally, there is a noticeable difference in efficiency between incorporation of NaN^OTP (and NaO^NTP) against ImO^N (and ImN^O) in the template and that of ImO^NTP (and ImN^OTP) against NaN^O (and NaO^N). Thus, the former is ∼1 order of magnitude less effective relative to the latter. The Im bases can be considered as ring-expanded analogues of purine toward the minor groove direction, while the Na bases are ring-expanded analogues of pyrimidine toward the major groove direction. In general, reaction by DNA polymerase tolerated steric repulsion in the major groove site in the template duplex,[7] whereas steric repulsion in the minor groove site in the template duplex appeared to have an adverse effect.[8] Thus, our results would seem to agree with these previous observations.

As described above, KF incorporated the noncanonical 5′-triphosphates against the complementary base in the templates. These recognitions would arise from the four H-bonds and also the shape complementarity of the Im:Na pair, which is similar to the purine:pyrimidine base pair. To confirm this observation, we next investigated how other possible base pairs are recognized by KF. Thus, one can imagine base pairs consisting of four H-bonds in ImN^O:ImO^N and NaN^O:NaO^N, although the shape complementarity of Im:Na pair is broken in these pairs (Figure S3). Furthermore, base pairs consisting of three H-bonds in ImN^O:NaN^O and ImO^N:NaO^N can also be postulated. However the enzymatic recognition of these possible base pairs was negligible in all cases as shown in lanes 3, 4, 6, and 9 of Figure 3. Similar results were also observed when NaN^O and NaO^N were introduced in the template (Figure S4).

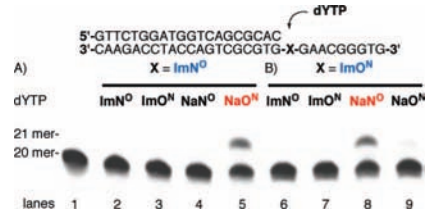

Figure 3. Single nucleotide insertion by Klenow fragment (selectivity toward noncanonical dNTPs). (A) Incorporation of dYTP against ImN^O in template. (B) Incorporation of dYTP against ImO^N in template. Experimental details are described in the Supporting Information.

In summary, we have investigated how thermally stable ImN^O:NaO^N and ImO^N:NaN^O pairs are recognized by KF. Although dATP and ImO^NTP were incorporated against NaN^O in the template, these complementary base pairs, especially the ImN^O:NaO^N pair, were recognized selectively by KF. This selectivity of these noncanonical pairs is considered to be due to the four H-bonds between the nucleobases and the shape complementarity of the Im:Na pair similar to the purine:pyrimidine base pair. To the best of our knowledge, this is the first example of enzymatic recognition of base pairs possessing four H-bonds. Our results would be a contribution toward developing alternative stable base pairs to expand the genetic code and explore the synthetic biology.

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Supporting Information Available: Synthesis of noncanonical nucleoside 5′-triphosphates and conditions applied in enzymatic incorporation. This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) The gel mobilities of lanes 7 and 12 differ from those of lane 8. However, this is due to the effect of the Im and Na nucleotide units in the resulting sequences. This was confirmed by comparison with separately prepared 21-mer sequences containing the Im and Na nucleotide units.
(6) For examples: (a) Guo, M.; Hildbrand, S.; Leumann, C. J.; McLaughlin, L. W.; Waring, M. J. *Nucleic Acids Res.* **1998**, *26*, 1863–1869. (b) Hendrickson, C. L.; Devine, K. G.; Benner, S. A. *Nucleic Acids Res.* **2004**, *32*, 2241–2250.
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