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Binding of hairpin pyrrole and imidazole polyamides to DNA: relationship between torsion angle and association rate constants

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ABSTRACT

N-methylpyrrole (Py)-N-methylimidazole (Im) polyamides are small organic molecules that bind to DNA with sequence specificity and can be used as synthetic DNA-binding ligands. In this study, five hairpin eight-ring Py–Im polyamides 1–5 with different number of Im rings were synthesized, and their binding behaviour was investigated with surface plasmon resonance assay. It was found that association rate (k_a) of the Py–Im polyamides with their target DNA decreased with the number of Im in the Py–Im polyamides. The structures of four-ring Py–Im polyamides derived from density functional theory revealed that the dihedral angle of the Py amide carbonyl is 14~18°, whereas that of the Im is significantly smaller. As the minor groove of DNA has a helical structure, planar Py–Im polyamides need to change their conformation to fit it upon binding to the minor groove. The data explain that an increase in planarity of Py–Im polyamide induced by the incorporation of Im reduces the association rate of Py–Im polyamides. This fundamental knowledge of the binding of Py–Im polyamides to DNA will facilitate the design of hairpin Py–Im polyamides as synthetic DNA-binding modules.

INTRODUCTION

N-Methylpyrrole (Py)-N-methylimidazole (Im) polyamides are small organic molecules that can recognize specific DNA sequences in the minor groove of B-form DNA, according to DNA recognition rules (1,2). Py favours T, A and C bases, whereas Im favours a G base. A lone electron pair on N-3 in Im forms a hydrogen bond with the 2-amino hydrogen of guanine (G). Thus, antiparallel pairings of Im/Py and Py/Im specify G/C and C/G, respectively, and antiparallel pairings of Py/Py specify A/T or T/A degenerately (1,2). Aliphatic β-alanine (β) can be substituted for Py. It has been used effectively when molecules have more than five consecutive Py or Im residues, by adjusting the pitch between amide bonds of Py–Im polyamides and the accepting residue of the minor groove. Antiparallel pairings of Py/β and β/Py specify A/T or T/A degenerately, and antiparallel pairings of Im/β and β/Im specify G/C and C/G, respectively (3,4).

As Py–Im polyamides can bind to DNA with sequence specificity comparable with DNA-binding proteins, they can be substituted for the DNA-binding domain of a transcription factor. Py–Im polyamides that can bind to a promoter region have been designed to inhibit gene expression (5–9). Furthermore, Py–Im polyamides have been conjugated with a peptide or a small organic molecule to create synthetic transcriptional activators that stimulate gene expression (10–13). The dissociation equilibrium constants (K_Ds) of these Py–Im polyamides with their target DNA sequences were extensively determined, by DNase I footprinting, by the Deriv group. However, only a few of their corresponding association rate constants and dissociation rate constants have been reported (14–16). It may be crucial for the design of a synthetic DNA-binding module to determine not only K_Ds...
MATERIALS AND METHODS

General methods

The following abbreviations apply: Fmoc, fluorenylmethoxycarbonyl; DMSO, dimethyl sulphoxide; TFA, trifluoroacetic acid; β, β-alanine; γ, γ-aminobutyric acid; Py, N-methylpyrrole; Im, N-methylimidazole; Dp, N, N-dimethyl-1,3-propylamine.

Electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) was carried out on a BioTOF II (Bruker Daltonics) mass spectrometer to determine the molecular weight of Py–Im polyamides 1–5.

Polyamide synthesis

Py–Im polyamides 1–5 were synthesized in a stepwise reaction using a previously described Fmoc solid-phase protocol (21). Syntheses were performed using a pioneer peptide synthesizer (PSSM-8, Shimadzu) with a computer-assisted operation system on a 36 μM scale (100 mg of Fmoc-β-alanine Wang resin). After the synthesis, Dp was mixed with the resin for 4 h at 55°C and the mixture was shaken at 550 r.p.m. to detach the Py–Im polyamides from the resin. Purification of Py–Im polyamides 1–5 was performed using a high-performance liquid chromatography (HPLC) PU-2080 Plus series system (JASCO), using a 10 x 150 mm ChemcoPak Chemocbond 5-ODS-H reverse-phase column in 0.1% TFA in water, with acetonitrile as eluent, at a flow rate of 3 ml/min and a linear gradient elution of 20–60% acetonitrile >20 min, with detection at 254 nm. Collected fractions were analysed by ESI-TOFMS.

SPR assay

All SPR experiments were performed on a BIACORE X instrument at 25°C as described previously (21,22).

RESULTS AND DISCUSSION

Hairpin eight-ring Py–Im polyamide synthesis

To investigate the binding properties of Py and Im in hairpin Py–Im polyamides, we designed and synthesized five hairpin eight-ring Py–Im polyamides 1–5 (Figure 1) by the Fmoc-chemistry solid-phase synthesis method. Two β-alanines were attached to the N-terminal of 1–5 (Figure 1) for the optional construction of a fluorescence Py–Im polyamide conjugate (Han et al., unpublished data). We purified 1–5 by reverse-phase HPLC, and then confirmed that the purity of 1–5 was >95% by analytical HPLC and ESI-TOFMS. The β-Dp linker at the

<table>
<thead>
<tr>
<th>Py–Im polyamide</th>
<th>$K_D$ (10^-9 M)</th>
<th>$K_a$ (10^4 M^-1 s^-1)</th>
<th>$k_d$ (10^-2 s^-1)</th>
<th>$\Delta G^{\circ}$ (kcal/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5 ± 0.50</td>
<td>150 ± 29</td>
<td>0.39 ± 0.12</td>
<td>-11.7</td>
</tr>
<tr>
<td>2</td>
<td>3.7 ± 2.3</td>
<td>120 ± 17</td>
<td>0.43 ± 0.25</td>
<td>-11.5</td>
</tr>
<tr>
<td>3</td>
<td>36 ± 10</td>
<td>28 ± 24</td>
<td>0.95 ± 0.70</td>
<td>-10.2</td>
</tr>
<tr>
<td>4</td>
<td>48 ± 4.7</td>
<td>30 ± 4.5</td>
<td>1.4 ± 0.08</td>
<td>-10.0</td>
</tr>
<tr>
<td>5</td>
<td>54 ± 9.9</td>
<td>13 ± 2.9</td>
<td>0.64 ± 0.13</td>
<td>-9.9</td>
</tr>
</tbody>
</table>

$^a$Closed circle and open circle indicate Im and Py, respectively.

$^b$$\Delta G^{\circ}$ values are calculated from the equation, $\Delta G^{\circ} = -RT\ln (1/K_D)$, where R is universal gas constant, 1.987cal/M•K; T is absolute temperature in a unit of K, here 298.15 K.
C-terminal has ~100-fold steric preference for A relative to G. Based on the recognition rule of polyamides, the target DNA sequences of 1–5 are 5'-WWWWWW-3', 5'-WWWWCW-3', 5'-WWWGCV-3', 5'-WWCGCW-3' and 5'-WGCGCW-3', respectively, and we prepared five 5'-biotinylated hairpin DNAs (ODN1–5) (Figure 2). However, because of two β-alanines attached to the N-terminal of 1–5, steric hinderance between the tails of 1–5 and the DNA minor groove may suppress the steric preference for A relative to G. To characterize the effect of the two β-alanines, we also prepared five 5'-biotinylated hairpin DNAs (ODN6–10) (Supplementary Figure S1).

**SPR assay**

To measure $K_D$, $k_a$ and $k_d$ values of 1–5 for their target DNA, we performed SPR assays as described in the ‘Materials and Methods’ section. The five 5'-biotinylated hairpin DNAs (ODN1–5) (Figure 2) that include the target DNA sequences were immobilized on a streptavidin-coated sensor chip, and the Py–Im polyamide solutions were injected. As shown in Figure 3, SPR sensorgrams were obtained, and the kinetic binding parameters $K_D$, $k_a$ and $k_d$ were determined (Table 1). The $K_D$s of 1–5 increased with an increase in the number of Im as follows: 2.5 x 10^{-9}, 3.7 x 10^{-9}, 3.6 x 10^{-8}, 4.8 x 10^{-8} and 5.4 x 10^{-8} M, respectively. Interestingly, the binding affinities of 1–4 were 22, 15, 1.5 and 1.1-fold, respectively, over that of 5. Of importance was that the $k_d$ values of 1–5 were comparable with each other (0.0039–0.014 s^{-1}) (Table 1), whereas the $k_a$ values of 1–5 were 1.5 x 10^{6}, 1.2 x 10^{6}, 2.8 x 10^{5}, 3.0 x 10^{5} and 1.3 x 10^{5} M^{-1}s^{-1}, respectively (Table 1). These results indicate that the association rate of the Py–Im polyamides with their target DNA decreased as the number of Im in the Py–Im polyamides increased. However, once 1–5 bound to their target DNA...
DNAs, the dissociation rates of the Py–Im polyamides from the respective complexes were comparable with each other. We also determined the free energy change (ΔG°, kcal/M) from the K_D on the formation of the Py–Im polyamides 1–5/DNA complexes (Table 1). The ΔG° values of 1/ODN1, 2/ODN2, 3/ODN3, 4/ODN4 and 5/ODN5 were −11.7, −11.5, −10.2, −10.0 and −9.9 kcal/M, respectively.

We also prepared five 5'-biotinylated hairpin DNAs (ODN6–10) (Supplementary Figure S1) to characterize the effect of the two β-alanines, and as shown in Supplementary Figure S2 and Supplementary Table S1, we determined the kinetic binding parameters K_D, k_a and k_d. As described earlier in the text, the K_D values of 1–5 also increased with an increase in the number of Im as follows: 6.1 × 10^{-9}, 1.3 × 10^{-8}, 2.5 × 10^{-8}, 1.1 × 10^{-7} and 2.5 × 10^{-7} M, respectively, and the binding affinities of 1–4 were 41, 19, 10 and 2.3-fold, respectively, over that of 5. The binding affinities of 1–5 to ODN6–10 were reduced 2.3, 3.5, 0.72, 2.3 and 4.6-fold, respectively, compared with those to ODN1-5 (Table 1 and Supplementary Table S1), whereas the k_d values of 1–4 were 1.8 × 10^6, 1.8 × 10^6, 5.1 × 10^5, 1.8 × 10^5 and 8.9 × 10^4 M^{-1}s^{-1}, respectively (Supplementary Table S1). The ΔG° values of 1/ODN6, 2/ODN7, 3/ODN8, 4/ODN9 and 5/ODN10 were −11.2, −10.8, −10.4, −9.5 and −9.0 kcal/M, respectively.

Previously, Crothers and coworkers reported the k_a and k_d values of ImPyPyPy-γ-ImPyPyPy-β-Dp, and the k_d value was 0.002 s^{-1}, which is consistent with our data. However, the k_a value was 7.0 × 10^7 M^{-1}s^{-1} and 46-fold times higher than that of 1. Dervan and coworkers (24) suggested that the I_m located at the C-terminal end of each four-ring Py–Im polyamide subunit is somehow less capable of strong hydrogen bond formation than the N-terminal residues. Therefore, the binding affinity of ImPyPyPy-γ-ImPyPyPy-β-Dp may be relatively high, like that of ImImPyPy-γ-ImImPyPy-β-Dp as discussed later in the text.
indicate that a large conformational change is necessary for Im residues on DNA binding. The fact that the angles of the two ring-to-amide bonds in each Py and Im ring were 147° and 138°, respectively, which indicates that Py has less curvature, and Im has too much curvature. This also suggests that the conformational change of Im residues could lead to a match to the regular B-form DNA, and 5 requires more energy than the other four Py–Im polyamide 1–4 to change the structure for binding to the target DNA, resulting in the slowest association rate of 5 with the target DNA among 1–5.

Dervan and coworkers also reported other Py–Im polyamides that recognize the GC-rich sequences 5'-GGGG-3' and 5'-GGGC-3' (24,28). In the case of the Py–Im polyamide that recognizes 5'-GGGG-3', the association equilibrium constant ($K_A$) is $2.8 \times 10^7$ M$^{-1}$ (The $K_D$ is $3.6 \times 10^{-8}$ M) (24). The $K_A$ of Py–Im polyamides that recognize 5'-GGGW-3', 5'-GGWW-3' and 5'-GWWW-3' have been determined as $3.7 \times 10^8$, $5.0 \times 10^8$ and $3.5 \times 10^9$ M$^{-1}$, respectively (28–31). Among these four Py–Im polyamides, the association equilibrium constant decreased as the number of Im in the Py–Im polyamides increased. However, the $K_A$ of the Py–Im polyamide that recognizes 5'-GGCC-3' was $9.7 \times 10^9$ M$^{-1}$ (24), which is higher than that of the Py–Im polyamide that recognizes 5'-GWWW-3'. The $K_A$ of the Py–Im polyamide that recognizes 5'-GGGW-3' was determined to be $4.0 \times 10^9$ M$^{-1}$ (32), which is similar to that of 5'-GGGW-3'. The Py–Im polyamide that recognizes 5'-GGGC-3' may be an exceptional Py–Im polyamide, like ImPyPyPy-$\gamma$-ImPyPyPy-$\beta$-Dp that recognizes 5'-GWWC-3' as described previously. However, the association equilibrium constant of the
other three Py–Im polyamides decreased as the number of Im in the Py–Im polyamides increased. Our calculated structural data indicate that ImPyPyPy is less curved compared with PyPyImPy (Figure 4B and C). These results suggest that not only the number of Im, but also the position of Im influences the Py–Im polyamide structure and the association rate of the hairpin eight-ring Py–Im polyamide to the target DNA.

As reported previously, replacement of Py with an aliphatic β-alanine can increase binding affinity and provide flexibility in the polyamide structures, and the binding affinity of Im-β-ImPy-γ-Im-β-ImPy-β-Dp that recognizes 5’-GCGC-3’ was 100-fold over that of ImPyImPy-γ-ImPyImPy-β-Dp (3). Measurement of $K_D$, $k_a$ and $k_d$ values of Py–Im polyamides containing Py/β and/or Im/β pairs is important for the next step of Py–Im
polyamide design. We have also replaced two Py in 5 by β-alanine, resulting in formation of β-β-Im-β-ImPy-γ-Im-β-ImPy-β-Im-Py, and we measured the $K_D$, $k_a$ and $k_d$ of β-β-Im-β-ImPy-γ-Im-β-ImPy-β-Im-Py. Interestingly, the $k_a$ and $k_d$ of β-β-Im-β-ImPy-γ-Im-β-ImPy-β-Im-Py were improved by ~10-fold, compared with those of 5 for ODN5 or ODN10 (Y.-W. Han et al., unpublished data). Further analysis of Py–Im polyamides containing Py/β and/or Im/β pairs is now in progress.

CONCLUSION
In this study, using SPR assays, we measured the $K_D$, $k_a$ and $k_d$ of Py–Im polyamides 1–5 to characterize Py and Im in hairpin Py–Im polyamides in more detail. Because $k_a$ and $k_d$ of some transcription factors have been determined and were contingent on the respective transcriptional factors, the measurement of $k_a$ and $k_d$ of Py–Im polyamides is also crucial for the design of a Py–Im polyamide as a synthetic DNA-binding module of a transcription factor. SPR data demonstrated that the $k_d$ values of 1–5 were between 0.0039 and 0.014 s$^{-1}$. The $k_a$ values of the Py–Im polyamides decreased as the number of im in the Py–Im polyamides increased. DFT calculations suggest that an increase in planarity, induced by the incorporation of Im, reduced the association rate of Py–Im polyamides. These data also demonstrated that the DNA binding kinetics of Py–Im polyamides were improved by C24–C24.

We synthesized Py–Im polyamide 1–5 which contained two β-alanines at the N-terminal in this study, and SPR data also demonstrated that the β-Dp linker at the C-terminal of 1–5 had a slight steric preference for A•T or T•A relative to G•C or C•G.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–4.

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Conflict of interest statement. None declared.

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