Development of Thiol-Responsive Amide Bond Cleavage Device and Its Application for Peptide Nucleic Acid-Based DNA Releasing System

Akira Shigenaga,* Jun Yamamoto, Hiroko Hirakawa, Keiji Ogura, Nami Maeda, Ko Morishita, Akira Otaka*
Institute of Health Biosciences and Graduate School of Pharmaceutical Sciences, The University of Tokushima
Development of Thiol-Responsive Amide Bond Cleavage Device and Its Application for Peptide Nucleic Acid-Based DNA Releasing System

Akira Shigenaga,* Jun Yamamoto, Hiroko Hirakawa, Keiji Ogura, Nami Maeda, Ko Morishita, and Akira Otaka*

Institute of Health Biosciences and Graduate School of Pharmaceutical Sciences, The University of Tokushima, Shomachi, Tokushima 770-8505, Japan.

ashige@ph.tokushima-u.ac.jp (AS), aotaka@ph.tokushima-u.ac.jp (AO)

Abstract—To develop a thiol-responsive DNA releasing system, a thiol-responsive amino acid capable of inducing an amide bond cleavage in the presence of a thiol was developed. It was successfully combined with peptide nucleic acid (PNA), and thiol-induced release of DNA from the thiol-responsive PNA/DNA complex was observed.

In the field of gene therapy and genetic recombination, development of a non-viral methodology for controlled delivery of DNA or RNA to prevent potential infection resulting from the use of viral vectors has been attracting increasing attention.1 In this context, release of an entrapped or encapsulated nucleic acid by a non-viral vector only after entering target cells is indispensable. An intracellular reductive environment, mainly due to the presence of thiols like glutathione, is one of the most attractive triggering substances for intracellular DNA/RNA releasing systems2 because the concentration of thiols is significantly different between the intracellular and extracellular environments (concentration of glutathione: 0.2 to 10 mM in cytoplasm; 2 µM in plasma).3 Previously, we reported a stimulus-responsive peptide which can induce a processing (peptide bond cleavage) reaction after stimulus-induced removal of a phenolic protective group followed by lactonization of the trimethyl lock moiety.4,5 In this paper, we report the development of a thiol-responsive amino acid and its application for a thiol-responsive DNA releasing system.

It is known that a nitrobenzenesulfonyl amide and ester can be easily cleaved in the presence of thiol,6 and thiol-induced cleavage of an aryl nitrobenzenesulfonate has been successfully applied as a quantitative thiol probe.7 With this in mind, we designed thiol-responsive peptide nucleic acid (PNA)8 possessing a p-nitrobenzenesulfonyl (pNs) group on the phenolic hydroxyl group of the stimulus-responsive amino acid (Scheme 1). Addition of PNA 1 to a solution of complementary DNA should induce hybridization to form a stable PNA/DNA double strand. In the presence of thiol, removal of the pNs group followed by a lactonization of the trimethyl lock moiety should cause fragmentation of PNA 1 at the C-terminal position of the thiol-responsive amino acid releasing the complementary DNA.

First, we attempted to synthesize model peptide 2, possessing a thiol-responsive amino acid, to examine its reactivity and selectivity against thiol. Scheme 2 shows the synthesis of thiol-responsive model peptide 2. The phenolic hydroxyl group on substrate 3b was sulfonylated with a p-nitrobenzenesulfonyl chloride in the presence of K2CO3 to afford pNs derivative 4. The TBS group on 4 was removed under acidic conditions to afford alcohol 5, and it was oxidized with PCC to give aldehyde 6. After subsequent oxidation of aldehyde 6 with NaClO2, followed by Boc removal by the action of TFA, the generated amine was protected with the Fmoc group to afford Fmoc-protected thiol-responsive amino acid 7. The total yield of Fmoc derivative 7 amounted to 64% over 6 steps beginning from phenol 3. Finally, incorporation of amino acid derivative 7 into a peptide by Fmoc solid phase peptide synthesis (Fmoc SPPS) afforded thiol-responsive model peptide 2.

To examine the reactivity and selectivity of model peptide 2 against thiol, we tested the processing reaction outlined in Scheme 3. Diastereomerically purified peptide 2 (0.02% w/v) in 30% w/v acetonitrile/phosphate buffer (20 mM, pH 7.6 or 9.0) was incubated with or without 0.1% w/v nucleophiles including thiol and amine at 37 °C for 24 h. The reaction progress was monitored by HPLC, and the resulting peptides were characterized by electrospray ionization mass spectrometry (ESI-MS). Yields of processing products were calculated based on peak areas in the HPLC chart, and the results are summarized in Table 1. In the presence of sodium 2-mercaptoethanesulfonate 11,10 processing products 8, 8' (an epimer of 8 at a carbon
The yield of processing observed because lactonization was possibly faster than reactions, 7.6 and 9.0 (entries 1 and 2, respectively). In these steps, the introduction of the electron withdrawing group on Ns group or treatment of peptide 2 with glutathione S-transferase is in progress. When peptide 2 was incubated without nucleophile or with a taurine as an amine nucleophile, the processing products were not detected (entries 3 and 4). These results suggest that the processing reaction of model peptide 2 occurs thioly-selectively.

Next, we monitored the time course of the thiol-responsive processing reaction of peptide 2 in the presence of 0.1% w/v thiol 11 at pH 9.0 (Figure S1). After 24 h of incubation at 37 °C, the yield of processing products almost reached a plateau. It might be caused by oxidative decomposition of thiol 11 under basic conditions to generate a disulfide by-product. In order to complete the processing reaction, we tried stepwise addition of thiol in the two-portion manner described below. Peptide 2 (0.02% w/v) in 30% v/v acetonitrile/phosphate buffer (pH 9.0, 20 mM) was incubated with 0.1% w/v thiol 11 at 37 °C for 24 h, and additional 0.1% w/v thiol 11 was added to the reaction mixture. It was incubated at the same temperature for an additional 24 h, and the reaction progress was monitored by HPLC (Figure 1). Whereas the substrate remained after incubation with 0.1% w/v thiol 11 at 37 °C for 24 h (Figure 1b), substrate 2 completely disappeared after stepwise addition of thiol 11 to afford processing products 8, 8′ and 9 (Figure 1c). When 0.2% w/v thiol 11 was added in one portion, the reaction did not finish within 48 h of incubation, and the yield of the processing products was 76%. Therefore we decided to use the two-portion protocol for a thiol treatment in subsequent experiments.

These results encouraged us to develop a thiol-responsive PNA. PNA 12 depicted in Scheme 4 was designed to possess a Gly-thiol-responsive amino acyl residue with length similar to that of thymine derived thiol residue (Figure 2). Additionally, a lysine residue was incorporated, which was expected to provide high solubility in water and to interact with negatively charged DNA. Fmoc SPPS using PyBOP (benzotriazol-1-yl)oxy)trispyrrolidinophosphonium hexafluorophosphate), HCTU (O-(6-chlorobenzotriazol-1-yl)-N,N′,N″-tetramethyluronium hexafluorophosphate), or HATU (O-(7-azabenzotriazol-1-yl)-N,N′,N″-tetramethyluronium hexafluorophosphate) allowed PNA 12 to be synthesized. Then we examined a thiol-responsive processing reaction of PNA 12 in phosphate buffer (pH 9.0) (Scheme 4). After treatment with thiol 11 in the two-portion manner, PNA 12 completely disappeared and processing products 13 and 14 were obtained in good purity (Figure S2). Next we examined the ability of PNA 12 to hybridize with complementary DNA and to release the DNA from the complex by treatment with a thiol.

Melting temperature (Tm) of PNA or DNA/DNA complex was measured in phosphate buffer (10 mM phosphate, 100 mM NaCl, pH 9.0); the results are summarized in Table 2. As a thiol-treated sample (depicted as “thiol +” in table 2), hybridized nucleic acids were incubated with 0.1% w/v of sodium 2-mercaptopethanesulfonate 11 at 37 °C for 24 h, and were subsequently incubated with additional thiol 11 (0.1% w/v) at the same temperature for 24 h. Melting curves were recorded using a fluorescence spectrometer (λem=525 nm, λex=600 nm) in the presence of ethidium bromide. In the absence of thiol, the melting curve of PNA 12/DNA(A9) complex was observed under these conditions, and Tm was estimated as 25.0 °C. (DNA(N9) refers to 5′-d(NNNNNN)-3′). As expected, Tm of PNA 12/DNA(A9) complex was lowered in the presence of thiol (entries 1 and 2). In contrast, DNA(T9)/DNA(A9) hybridization was not affected by thiol treatment (entries 3 and 4). Furthermore, Tm of PNA 12/DNA(A9) complex was higher than that of corresponding DNA(T9)/DNA(A9) complex (entries 1 and 3). These results suggest that PNA 12 is potentially applicable to a thiol-responsive DNA releasing system.

In conclusion, we developed a thiol-responsive self-processing amino acid which induces an amide bond cleavage reaction after treatment with thiol. With its successful combination with a peptide nucleic acid, thiol-responsive PNA was generated. Melting temperature experiments clarified that thiol-responsive PNA 12 can bind to complementary DNA(A9), and the Tm of the thiol-responsive PNA 12/DNA(A9) complex was drastically decreased by treatment with thiol. These results suggest that thiol-responsive PNA is potentially applicable as a DNA binding moiety of a thiol-responsive gene delivery system. Development of the thiol-responsive PNA with sequence specificity and high sensitivity to thiols, and its application for gene delivery system are in progress.

Acknowledgments

We thank Dr. M. Nishida (Osaka University) for valuable discussion. This research was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI), Takeda Science Foundation, The Science and Technology Foundation of Japan, and Nagase Science and Technology Foundation.

References


10. Because glutathione and its disulfide derivative make HPLC chart complicated, thiol 11 was used as a model thiol.

12. Thiol-responsive PNA 12: Analytical HPLC condition (Cosmosil 5C18-AR-II analytical column (Nacalai Tesque, 4.6×250 mm, flow rate 1 mL/min)): linear gradient of solvent B in solvent A, 1 to 50% over 30 min. Retention time=22.0 min. MS (ESI-IT) calc. for C115H152N38O40S ([M+2H]2+): 1369.1, found; 1369.1.


Legends

Scheme 1. Design of thiol-responsive peptide nucleic acid (PNA).

Scheme 2. Reagents and conditions. (i) p-nitrobenzenesulfonyl chloride (pNsCl), K2CO3, acetone, reflux, quant.; (ii) AcOH, H2O, THF, quant.; (iii) PCC, CH2Cl2, 91%; (iv) NaClO2, NaH2PO4, 2-methyl-2-butene, tert-ButOH, H2O, acetone; (v) HCl, AcOEt; (vi) FmocOSu, Na2CO3, H2O, MeCN, 70% (3 steps); (vii) Fmoc SPPS; (viii) TFA/triethylsilane/H2O=95/2.5/2.5 v/v/v.

Scheme 3. Reagents and conditions. (i) 0.1% w/v nucleophile, phosphate buffer (20 mM), 30% v/v MeCN, 37 °C, 24 h under Ar. Peptide 8’ is an epimer of 8 at the asterisked carbon.

Scheme 4. Reagents and conditions: (i) 0.1% w/v thiol 11, phosphate buffer (pH 9.0, 20 mM), 30% v/v MeCN, 37 °C, 24 h under Ar. Subsequent addition of 0.1% w/v thiol 11, 37 °C, 24 h under Ar, as per the two-portion manner.

Figure 1. HPLC profiles (a) before treatment with thiol 11, (b) after 24 h incubation with 0.1% w/v thiol 11 at 37 °C under Ar, and (c) after 24 h incubation with 0.1% w/v thiol 11 at 37 °C under Ar, followed by subsequent incubation with additional 0.1% w/v thiol 11 at 37 °C for 24 h under Ar. Peptides were detected by UV absorbance at 220 nm. Asterisked peak is not a peptidic compound.

Table 1. Effects of pH and nucleophile on the processing reaction of peptide 2 as shown in Scheme 3.

Table 2. Results of melting temperature experiments.
Gly-thiol-responsive amino acyl residue

t residue
<table>
<thead>
<tr>
<th>Entry</th>
<th>Nucleophile</th>
<th>pH</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HS(CH₂)₂SO₃Na (11)</td>
<td>7.6</td>
<td>37</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>9.0</td>
<td>68</td>
</tr>
<tr>
<td>3</td>
<td>H₂N(CH₂)₂SO₃H</td>
<td>9.0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>none</td>
<td>9.0</td>
<td>0</td>
</tr>
<tr>
<td>Entry</td>
<td>Nucleic Acid</td>
<td>Thiol</td>
<td>$T_m$ (°C)</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>1</td>
<td>PNA 12/DNA(A9)</td>
<td>-</td>
<td>25.0</td>
</tr>
<tr>
<td>2</td>
<td>PNA 12/DNA(A9)</td>
<td>+</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>3</td>
<td>DNA(T9)/DNA(A9)</td>
<td>-</td>
<td>18.5</td>
</tr>
<tr>
<td>4</td>
<td>DNA(T9)/DNA(A9)</td>
<td>+</td>
<td>18.5</td>
</tr>
</tbody>
</table>

(a) Conditions: 4.0 µM concentration for each strand, 10 mM phosphate buffer (pH 9.0), 100 mM NaCl.
Thiol -: The sample was not treated with thiol 11.
Thiol +: The sample was treated with thiol 11 (0.1% w/v at 37°C for 24 h under Ar, followed by subsequent treatment with thiol 11 (0.1% w/v) again at 37°C for an additional 24 h under Ar. Melting curves were recorded in the presence of ethidium bromide ($\lambda_{ex}$=525 nm, $\lambda_{em}$=600 nm). $T_m$s are the average of two runs. DNA(N9) denotes 5’-d(NNNNNNNNN)-3’.