Heterogeneity of osmium oxidation efficiency at consecutive thymines†

Akiko Nomura and Akimitsu Okamoto*

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Consecutive thymine sequences were oxidized more efficiently with a mixture including potassium osmate, potassium hexacyanoferrate(III), and bipyridine than an isolated thymine, and, in particular, osmium oxidation at the 5′T of TT progressed much more rapidly, compared with those of other thymines.

Oxidative DNA damage has been extensively studied because of its significant involvement in gene mutation, aging, and cancer.[1] Oxidation of a nucleobase provides, for example, 8-oxoguanine[2] and imidazolone derivatives[3] from guanine and thymine glycol[4] and 5-formyluracil[5] from thymine. Thymine is known to be more easily oxidized by an extracellular oxidant osmium tetroxide than other nucleobases, and thus osmium oxidation is currently applied for thymine-targeting DNA sequencing.[6] However, different band intensities of each thymine, *i.e.*, different reactivities with osmium tetroxide, have been observed in gel electrophoresis analysis. The reason for the heterogeneity of the oxidation efficiency at thymines remains unclear; although involvement of the secondary structure of DNA was once proposed.[7] We herein report the osmium oxidation of single-stranded oligodeoxynucleotides (ODNs) containing consecutive thymine sequences. Osmium oxidation of the sequence in which two thymines were consecutive (TT) progressed more rapidly, compared with that of the sequence containing an isolated thymine, with the 5′T of TT showing higher reactivity than the 3′T.

We prepared 5′-^{32}P-labeled ODN sequences containing one or more thymines (Fig. 1a), and treated them with a mixture including potassium osmate and potassium hexacyanoferrate(III) as an activator, and bipyridine as a reaction-accelerating ligand (Fig. 1b).[8] The reaction products were incubated in hot piperidine and then analyzed with polyacrylamide gel electrophoresis (PAGE) (Fig. 1c). In a series of operations, an osmium-centered heterocyclic complex was formed at a thymine base on osmium oxidation,[9] and the ODN strands were fragmented *via* depyrimidination at the complexation site by alkaline cleavage to quantify the product. Osmium complexation proceeds rapidly at thymines and 5-methylcytosines in single-stranded ODNs, although the reaction is strongly suppressed in double-stranded ODNs.[8a] The starting ODNs in the reaction mixture were consumed depending on the reaction time. Interestingly, the reaction of ODN(GTTG) proceeded more than twice as fast as that of ODN(GTG) in the first 0.25 min of reaction (Fig. 1d). The half-lives ($t_{1/2}$) of ODN(GTG) and ODN(GTTG) were 86 and 13 s, respectively. The reaction observed for ODN(GTTTG) was faster than that of ODN(GTTG) ($t_{1/2}$ = 11 s). These sequence-dependent reaction rates suggest that consecutive thymine sequences have much higher reactivity towards osmium oxidation than an isolated thymine. In fact, the reaction rate of ODN(GTTG) was significantly faster

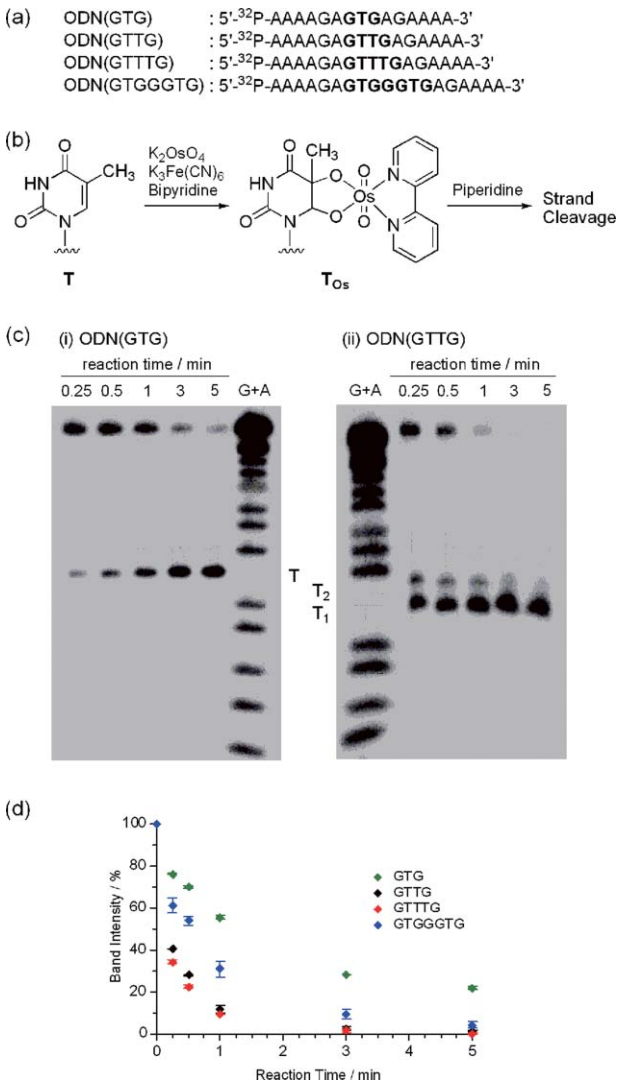

Fig. 1 Osmium oxidation of ODNs containing thymines. (a) Sequences of ODNs used in this study. (b) Osmium complexation at thymine and strand cleavage at the complexation site. (c) PAGE analysis of the oxidation products of ^{32}P-labeled ODNs. The ODNs were incubated at 0 °C for 0.25–5 min in a solution of 5 mM potassium osmate, 0.1 M potassium hexacyanoferrate(III), 0.1 M bipyridine, 1 mM EDTA in 0.1 M Tris-HCl (pH = 7.7), and 10% acetonitrile, and followed by hot piperidine treatment (90 °C, 20 min). "G + A", a Maxam–Gilbert G + A sequencing lane. (d) Time course of the band intensities of intact ODNs.

Advanced Science Institute, RIKEN (The Institute of Physical and Chemical Research), Wako, Saitama, 351-1098, Japan. E-mail: aki-okamoto@riken.jp; Fax: +81 48 467 9205; Tel: +81 48 467 9238

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compared with that of the ODN bearing two isolated thymine bases, ODN(GTGGGTG).

Another important feature observed in Fig. 1c is that the cleavage band of 5′T (T_1) of ODN(GTTG) was much stronger than that of 3′T (T_2). The cleavage observed for ODN(GTTTG) also showed a 5′ selectivity similar to that of ODN(GTTG): 5′T > centerT > 3′T.[10] In the case of ODN(GTTG), the cleavage band at T_2 appeared once, but the intensity decreased as time passed. The band almost converged to T_1 after 5 min of reaction. Based on the change in the intensities of the cleavage bands, we calculated the ratio of the reaction rates of thymines in ODN(GTTG). Because ODN(GTTG) used for PAGE analysis was labeled at the 5′-end, the band intensity at T_1 is attributed to both ODN($GT_{Os}TG$) and doubly reacted ODN($GT_{Os}T_{Os}G$), where T_{Os} denotes a thymine glycol–dioxoosmium(VI)-bipyridine ternary complex as a reaction product. Therefore, the equations for complexation at T_1 and T_2 can be set up as shown in Fig. 2a. Simulated curves correlated well with the observed band intensities when a higher reactivity for T_1 and a lower reactivity with the second osmium were assumed. The α value as the ratio of reaction rates of thymines (k_1/k_2) was 2.22, which means that the reactivity of T_1 is approximately twice that of T_2. On the other hand, the α value obtained by calculation for ODN(GT_1GGGT_2G) was estimated as 1.09 ($k_1 = (8.90 \pm 1.40) \times 10^{-3}$ s^{-1}), suggesting that the reaction rate at each thymine was not as rapid as that of a TT sequence and there is little difference in the reactivities of two isolated thymines. The α value of ODN(GTTG) indicates that the enhancement of the reactivity at 5′T is a unique feature observed for the TT sequence. Higher reactivity of 5′T was also observed in the osmium oxidation for a 3′-end-labeled ODN.[10] The ratio of oxidation rates at T_1 and T_2 of a 3′-end-labeled ODN was estimated as $\alpha = 1.80$, which is very close to the data of 5′-end-labeled ODN(GTTG).

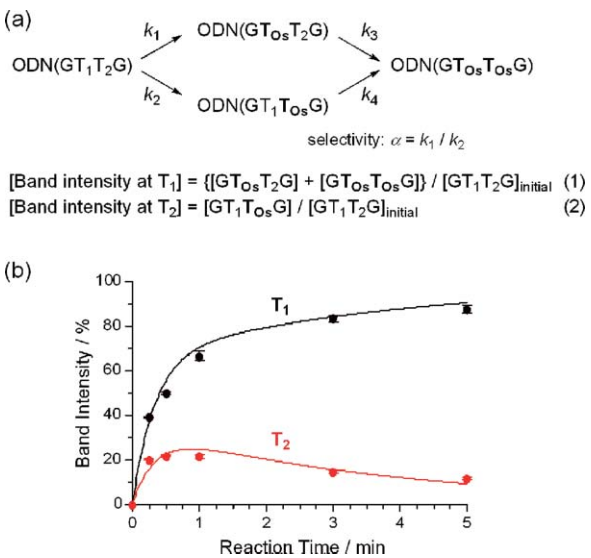

Fig. 2 Estimation of the rates of osmium oxidation at T_1 and T_2 of ODN(GTTG). (a) The normalized band intensities at the T_1 and T_2 sites expanded to eqn (1) and (2), respectively. (b) Change in the band intensities at T_1 and T_2. Closed circles show band intensities quantified from Fig. 1c. Solid lines show the predicted time-course of band intensities obtained by simulation. $k_1 = (3.61 \pm 0.20) \times 10^{-2}$ s^{-1}, $k_2 = (1.63 \pm 0.10) \times 10^{-2}$ s^{-1}, $k_3 = k_4 = (4.43 \pm 0.67) \times 10^{-3}$ s^{-1}, $\alpha = 2.22$, $r^2(T_1) = 0.983$, $r^2(T_2) = 0.912$.

The selective oxidation enhancement at 5′T was almost independent of the nature of the neighboring base of the TT sequences. The osmium oxidation of ODN(XTTX) (5′-^{32}P-AAAAGAXTTXAGAAAA-3′, X = G, A, or C) showed no significant differences in 5′T-selective reaction enhancement.[10] The result suggests that the 3′T of TT assists the 5′T-selective reaction enhancements. Observation of an increase in the oxidation efficiency at 5′T of TT was not limited to osmium oxidation. For example, the oxidation of thymine when potassium permanganate was employed, which provides a thymine glycol in a similar way to osmium dihydroxylation, also showed predominant oxidation at T_1 of ODN(GTTG).[10] The osmium oxidation rate of ODN(GTTG) was higher than those of ODN(GTG) and ODN(GTGGGTG), and the 5′T of ODN(GTTG) showed higher reactivity to osmate and permanganate than 3′T. This heterogeneity of oxidation efficiencies would be attributable to the accessibility of oxidants to thymines. The X-ray crystal structure of an osmium adduct of thymine showed that the osmium complex is located at the 3′ side of the thymine face because of formation of (5*R*,6*S*)-thymine glycol as a major product.[9] This structure shows that the oxidant approached to the target thymine from the 3′ side, thus the size of the base located at the 3′ side of the target thymine, *i.e.*, smaller thymine base or larger guanine base, would influence the accessibility of oxidants to thymines and result in oxidation efficiency different in each thymine.

The base located at the 3′ side of thymine affected the rate of osmium complex formation. Therefore, the effect of a pyrimidine base placed at the 3′ side of target thymine on the reactivity of the thymine on osmium complexation was investigated using ODN(GTPyG) (5′-^{32}P-AAAAGAGTPyGAGAAAA-3′, Py = T, C, 5-methylcytosine (M), or uracil (U)). The reactivity at the thymine of ODN(GTPyG), calculated from the cleavage band intensities on PAGE analysis (Fig. 3), greatly depended on the pyrimidine bases located at the 3′ side; the order of the reactivity (k at the underlined thymine) was G<u>T</u>TG ($k = (3.61 \pm 0.20) \times 10^{-2}$ s^{-1}) > G<u>T</u>UG ($(3.22 \pm 0.23) \times 10^{-2}$) ≥ G<u>T</u>MG ($(2.94 \pm 0.11) \times 10^{-2}$) > G<u>T</u>CG ($(2.20 \pm 0.66) \times 10^{-2}$) > G<u>T</u>G ($(8.19 \pm 1.44) \times 10^{-3}$). The difference of osmium complexation rate at 5′T caused by the difference of 3′Py was not large but significant. The reason why 3′T enhanced the reactivity of 5′T so much is still unclear: the methyl group at C5 and/or the carbonyl group of C4 of 3′Py might have worked as a bridgehead for the approach of oxidants to 5′T, or the oxidation potential of 3′Py[11] might have influenced the reactivity of 5′T like the 5′-selective oxidation observed at a two consecutive guanine sequence.[12]

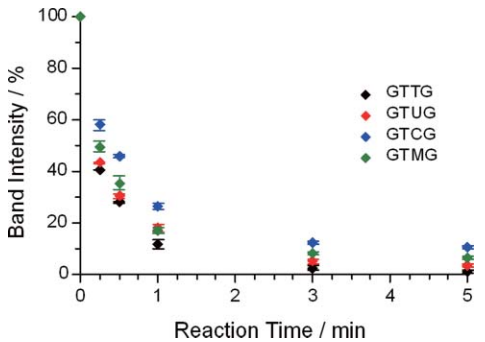

Fig. 3 Time course of the band intensities of intact ODN(GTXG).

Having established heterogeneity of osmium oxidation efficiency at consecutive thymines using model sequences, we investigated osmium oxidation of wild-type DNA sequences. Three single-stranded DNA fragments, 1085–1124 and 1275–1335 of pUC19 and 518–562 of pFOS1 were treated with the oxidant followed by hot piperidine. The band intensity in the PAGE analysis for strand cleavage at TT sequences was liable to be higher than for cleavage at the isolated thymine sequences (Fig. 4). In addition, cleavage at the 5′T of consecutive thymine sequences was stronger than for 3′T. Heterogeneity of osmium oxidation efficiency at consecutive thymines observed in model experiments was reproduced in wild-type DNA sequences.

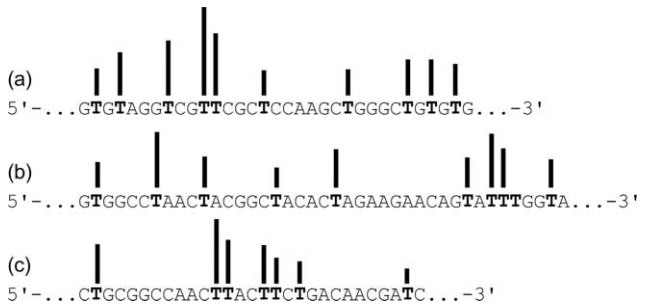

Fig. 4 DNA cleavage sites by osmium oxidation. (a) pUC19 (1085–1124) (Reaction time: 20 s); (b) pUC19 (1275–1335) (Reaction time: 10 s); (c) pFOS1 (518–562). (Reaction time: 5 s). The height of the bars in the histograms shows the relative band intensity at a given site.

We have reported in this paper the heterogeneity of the oxidation efficiency at thymines. A TT sequence was oxidized more efficiently than isolated thymines, and it is noteworthy that the oxidation rate at thymine was raised by the 3′-neighboring thymine. This research showed that consecutive thymine sequences result in the heterogeneity of the oxidation efficiency at thymines. Such a heterogeneous reactivity of thymines is a new class of sequence-selective pattern in DNA damage and mutagenesis. Thymine oxidation in a single-stranded DNA is a base conversion that gives us chemically and biologically important information, and also useful for structure-sensitive gene analysis. Further analysis on sequence-dependent reactivity of thymines and its mechanism would strongly support new designs for DNA-targeting drugs.

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