Novel Enzymatic Properties of DNA−Pt Complexes

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Received February 3, 2007; Revised Manuscript Received June 15, 2007

DNA−Pt complexes have shown novel enzymatic activity as a peroxidase similar to that of horseradish peroxidase in the colorimetric reaction with its substrate. The enzymatic activity of these complexes increased with increasing reaction time and pH in reaction solutions of DNA and $K_2[PtCl_4]$. This enhanced enzymatic activity was attributed to the increase in Pt conjugated to DNA in the complex. The enzymatic activity per unit mole of the DNA−Pt complex was significantly higher for complexes prepared with high molecular weight DNA because the enzymatic activity of the complex per repeat unit of DNA was almost constant for these complexes prepared under the same reaction conditions. All the DNA−Pt complexes in this study prepared with different DNA sequences (i.e., $[A]_{20}$, $[G]_{20}$, $[C]_{20}$, $[T]_{20}$, and $[AG]_{10}$) exhibited peroxidase enzymatic activity. These complexes showed good thermal stability as compared to native horseradish peroxidase.

Introduction

DNA has not played a role as a biocatalyst in evolutionary history, although RNA and proteins function as biocatalysts. There are nine known classes of natural ribozymes that catalyze phosphoester cleavage/formation or peptide bond forming reactions.[1] The ability of RNA to serve as a catalyst was first shown for the self-splicing group I intron of *Tetrahymena thermophila* and the RNA moiety of RNAse P.[2,3] An HIV ribozyme directed to the nucleolar compartment was reported to inhibit HIV replication successfully.[4] Recently, double-stranded RNAs (dsRNAs) that induced targeted degradation of complementary RNA sequences by a process known as RNA interference (RNAi) have also been developed.[2,5,6]

However, it was believed that it was impossible to form the double-helix structure of DNA into intricate active enzymatic sites. In addition, the chemical stability of DNA prevents self-modifying reactions.[7] However, recent developments in DNA engineering have enabled the creation of an artificial enzymatic ability in DNA (forming substances known as DNA enzymes, DNAzymes, or deoxyribozymes) for activities such as RNA cleavage[8−11] and DNA[12−15] and RNA modification.[16−18] A catalytic DNA molecule, Dz13, was reported to suppress vascular permeability and transendothelial emigration of leukocytes by targeting the transcription factor c-Jun.[11] An RNA cleaving phosphodiester-linked deoxyribozyme, targeting Egr-1 (an immediate early gene inducibly expressed in growth-quiescent fibroblasts exposed to serum), was found to inhibit endothelial expression of a fibroblast growth factor (FGF)-2 but not that of a vascular endothelial growth factor (VEGF) and consequently inhibited human breast carcinoma growth in nude mice.[16] Another type of DNAzyme was also reported as a signal amplification for DNA detection, when DNA was complexed with hemin, which expresses peroxidase characteristics.[19−22] DNA complexed with hemin can catalyze a chemical oxidation and generate a colorimetric output, which can be used in the detection of specific DNA such as M13 phage single-stranded DNA (ssDNA).[21]

Recently, we found that DNA−Pt complexes might have an enzymatic activity similar to peroxidase (unpublished data). A colorimetric reaction of substrate took place on immobilized DNA membranes in the absence of a secondary antibody conjugated with peroxidase during experiments quantifying adsorbed proteins on membranes using an enzyme-linked sorbent assay, ELISA (unpublished data). Because the immobilized DNA membranes were prepared from the reaction of DNA on immobilized Pt membranes,[23−25] it was thought that a DNA−Pt complex in aqueous solution would act as a peroxidase. Such a complex would be more chemically stable than a natural enzyme because the phosphodiester bonds of DNA are 100-fold more resistant to hydrolytic degradation than the peptide bonds of proteins.[7]

We report here optimal conditions (i.e., oligonucleotide sequence of DNA and pH) for the preparation of DNA−Pt complexes having oxidase enzymatic activity and compare their activity with that of horseradish peroxidase. We further compare the thermal stability of DNA−Pt complexes with that of the natural enzyme upon heat treatment.

Experimental Procedures

Materials. DNA (from salmon testes, D-1626 for dsDNA and D-7656 for ssDNA (M_w = 46 800 Da)), adenosine-5′-monophosphate sodium salt, and horseradish peroxidase (HRP, P-6782, 1000 units/mg) were purchased from Sigma Aldrich, Inc. (St. Louis, MO). Adenosine-5′-triphosphate disodium salt was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). DNA oligonucleotides (adenine (A)-5mer, -10mer, -15mer, -20mer, -25mer, guanine (G)-20mer, cytosine (C)-20mer, thymine (T)-20mer, and AG-20mer ($[AG]_{10}$)) were purchased from Sigma Genosys (Tokyo, Japan). Potassium tetrachloroplatinate (II) ($K_2[PtCl_4]$) was purchased from N.E. Chemcat Corp.

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10.1021/bm070137i CCC: $37.00
Published on Web 08/03/2007

(Shizuoka, Japan). TMB (3,3′,5,5′-tetramethylbenzidine) microwell peroxidase substrate systems were purchased from KPL (Gaithersburg, MD). Dulbecco's phosphate buffered saline (PBS, pH 7.0) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Tetraborate pH standard solution (028−-03205, pH 9.18) purchased from Wako Pure Chemicals Ltd. (Tokyo, Japan) and buffer prepared from 0.05 mol/L Na_2HPO_4 and 0.1 M NaOH (pH 11) were used to adjust the pH of the reaction solution of $K_2[PtCl_4]$ and DNA. TE buffer containing 5 mmol/L Tris-HCl and 0.5 mmol/L EDTA at pH 7.0 was used to dissolve the DNA−Pt complexes. Other chemicals, purchased from Tokyo Chemical Co. (Tokyo, Japan), were reagent grade and were used without further purification. Ultrapure water was used throughout the experiments.

Preparation of DNA−Pt Complexes. DNA was dissolved in a buffer solution of pH 7, 9, or 11 to give 500 mg/L DNA solutions. Potassium tetrachloroplatinate (II) ($K_2[PtCl_4]$) was dissolved in ultrapure water to yield an 8330 mg/L $K_2[PtCl_4]$ solution. A total of 100 μL each of the DNA and $K_2[PtCl_4]$ solutions was mixed and incubated at 25 °C for 24, 48, 72, 96, 120, and 144 h in the dark. A total of 20 μL of a 3 mol/L sodium acetate solution and 500 μL of ethanol (99.5%) were added to each DNA−Pt complex solution, which was then kept in a freezer at −20 °C for 24 h to precipitate the complex. After centrifuging the solution at 15 000 rpm (4 °C, 15 min), the supernatant was removed carefully, and 500 μL of 70% ethanol solution was added. The solution was centrifuged again in the same way for recrystallization, and the ethanol solution was removed from the DNA−Pt complex by decantation, which was finally dried in vacuum for 24 h at room temperature.

Characterization of DNA−Pt complex. The DNA−Pt complex was diluted to 100−200 pmol/μL in ultrapure water. Samples were prepared for mass analysis using the following matrices: 10 mg of 3-hydroxypicolinic acid (HPA) was dissolved with 200 μL of H_2O and 200 μL of acetonitrile and adjusted to 25 mg/mL. A total of 2 μL of sample solution (DNA−Pt complex solution) was mixed with 2 μL of the matrices solution on the sample holder and allowed to dry at room temperature. The mass analyses were performed with a MALDI-TOF mass spectrometer (AXIMA-CFRplus, Shimadzu, Japan), equipped with a nitrogen laser. The spectra were obtained in the negative or positive ion mode at 90−135 kV by summing 100 laser shots. Atomic analysis of the DNA−Pt complex was performed using XPS (ESCA-3400, Kratos Analytical Ltd., Kyoto, Japan).

Enzyme Reactions. HRP was dissolved in PBS (pH 7.0), and the DNA−Pt complex was dissolved in TE buffer (pH 7.0), to the appropriate concentrations. Next, 0.2 mL of an H_2O_2 solution containing the TMB substrate for peroxidase and the DNA−Pt complex was added to 0.2 mL of HRP solution or the DNA−Pt complex solution of each concentration in a 24-well tissue culture flask. The reaction solutions were incubated for 20 min at 37 °C. The buffer solution without containing HRP or the DNA−Pt complex and H_2O_2 solution containing the TMB substrate were also incubated in the same procedures as stated previously. The absorbance of the solution was measured at 450 and 595 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, Model 550) after 0.2 mL of a stop solution (1 mol/L H_3PO_4 solution) was injected into the enzyme reactions in the tissue culture flask. These measurements were carried out 4 times for each solution.

Results and Discussion

Characterization of DNA−Pt Complex. We first used MALDI-TOF mass spectroscopy to examine the formation of DNA−Pt complexes from the reaction between DNA and $K_2[PtCl_4]$. Figure 1 illustrates the mass spectra of DNA (A-5mer) and the DNA (A-5mer)−Pt complex prepared at pH 7 and 24 h reaction time. The molecular weight of A-5mer is 1536 Da, and the mother peak at around 1540 Da was found in the mass spectra of both DNA and DNA−Pt complex. Additional peaks at around 1800−1840 Da were also found in the spectra of the

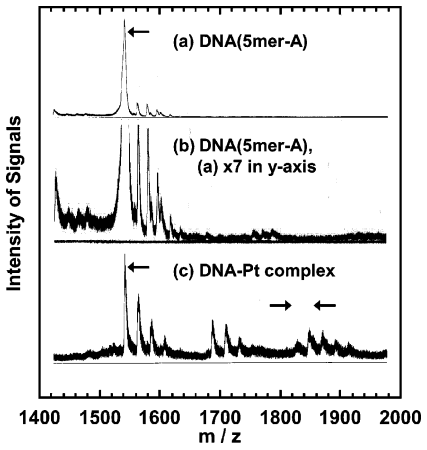

Figure 1. Mass spectroscopy of DNA and DNA−Pt complex measured by MALDI-TOF mass spectroscopy. 5mer-adenine oligomer was used as DNA (a and b). Spectrum of panel b is the spectrum of panel a multiplied by a factor of 7 in the *y*-axis. DNA−Pt complex was prepared at pH 7 and reaction time of 24 h (c).

Table 1. Atomic Concentration Ratio of DNA−Pt Complex Prepared Using ssDNA at pH 7, 9, and 11 and Reaction Times of 24 and 120 h

	reaction conditions		
sample	reaction time (h)	pH	P1s/Pt1s
DNA−Pt-1	24	7	139
DNA−Pt-2	24	11	45.7
DNA−Pt-3	120	7	126
DNA−Pt-4	120	9	56.5
DNA−Pt-5	120	11	33.9

complex, indicating the binding of −-$PtCl_3$ or −-$PtCl_4$ to DNA, whereas no peaks were observed in this region in the spectra of DNA. These results suggested that at least one platinum atom could possibly attach to DNA with a covalent bond.

XPS analysis was performed on DNA−Pt complexes prepared using ssDNA at pH 7, 9, and 11 and reaction times of 24 and 120 h. Table 1 shows the atomic mole fraction ($P1_S/Pt1_S$) of DNA in the DNA−Pt complexes. This indicated that one atom of Pt was conjugated per DNA repeat unit because there is only one atom of P in each repeat unit. It was found that the amount of Pt bound to DNA increased with an increasing reaction pH and time. However, each atom of Pt bound to more than 33.9 repeat units of DNA under the present reaction conditions from Table 1.

Enzymatic Activity of DNA−Pt Complex. The peroxidase activity of the complex prepared using $[AG]_{10}$ DNA at pH 7, 9, and 11 was investigated. The substrate selected was TMB, which is widely used as a substrate for horseradish peroxidase in diagnosis and ELISA bioassays.[26,27] Horseradish peroxidase and DNA−Pt reacted with TMB, showing a colorimetric reaction at the absorbance maximum of 450 nm (Figure 2). No oxidation reaction of TMB (no color change) was found in the TMB reagent solution and buffer solution without containing HRP or the DNA−Pt complex after the incubation in the present reaction conditions as found in the lanes of ssDNA and $K_2[PtCl_4]$ in Figure 2. The absorbance at 450 nm (A_{450}) minus the absorbance at 575 nm (A_{575}) was investigated as a function of HRP concentration in 1 mL of reaction solution containing TMB and 1000 units/mg of HRP (see Supporting Information). The calibration curve of the enzymatic activity could, therefore, be obtained from the absorbance at HRP concentrations from 0.03 to 14 unit/L (C_{HRP})

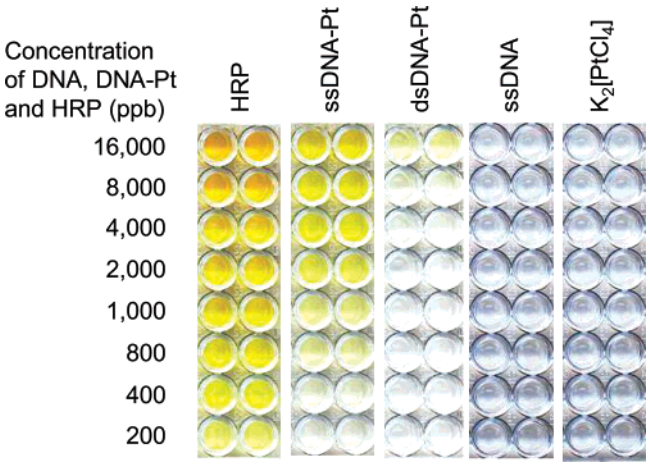

Figure 2. Enzymatic activity of HRP, DNA−Pt complex, DNA, and $K_2[PtCl_4]$. ssDNA−Pt complex was prepared using ssDNA at reaction time of 24 h and pH 7. dsDNA−Pt complex was prepared using dsDNA at reaction time of 24 h and pH 7.

$$A_{450} - A_{575} = 0.18622C_{HRP}/(\text{unit/L}) - 0.0015 \qquad (1)$$

The correlation coefficient (r) of eq 1 was found to be 0.999.

Figure 3 illustrates the peroxidase enzymatic activity of the DNA−Pt complex as a function of reaction time at pH 7, 9, and 11.

It was found that the DNA−Pt complex acted as a peroxidase, and this enzymatic activity increased with increasing reaction time and pH in the reaction solution containing DNA and $K_2[PtCl_4]$. Because the mole ratio of Pt to DNA in the DNA−Pt complex increased with increasing reaction time and pH in this reaction solution, as shown in Table 1, the increase in enzymatic activity of the DNA−Pt complex was attributed to the greater amount of Pt conjugated to the DNA in the complex. The enzymatic activity of the DNA−Pt complex originated from platinum bound to DNA because no peroxidase activity was found when using only DNA or $K_2[PtCl_4]$, as shown in Figure 2.

The effect of specific DNA sequences in these complexes on their enzymatic activity was investigated using the following DNA 20mer oligonucleotides: AG-20mer ($[AG]_{10}$), A-20mer, C-20mer, G-20mer, and T-20mer. Figure 4 illustrates the enzymatic activity of the complexes prepared from these 20mers at pH 7, 9, and 11 and 120 h reaction time. Complexes prepared at higher pHshowed a higher enzymatic activity. The DNA−Pt complexes prepared from AG-20mer and A-20mer showed a higher enzymatic activity than those prepared from C-20mer, G-20mer, and T-20mer, although all the complexes in this study showed peroxidase activity.

Platinum binding sites on DNA have been extensively studied for the antitumor drug cisplatin (*cis*-diamminedichloroplatinum [II]),[24] but the analogous binding sites on our DNA−Pt complexes are as yet unknown. Cisplatin has been reported to bind to the N7 atom of guanine; the N7 and N1 atoms of adenine; and the N3 atom of cytosine under neutral conditions.[28,29] Competitive reactions of the four nucleoside monophosphates with cisplatin have established the order of reactivity by Raman difference spectrophotometry as GMP > AMP ≫ CMP > UMP, confirming that N7 of GMP was the most preferred binding site.[28,29] Therefore, the binding structure and conformation of platinum from $K_2[PtCl_4]$ on DNA might be different from those from cisplatin on DNA because the enzymatic activity of the DNA−Pt complexes prepared from

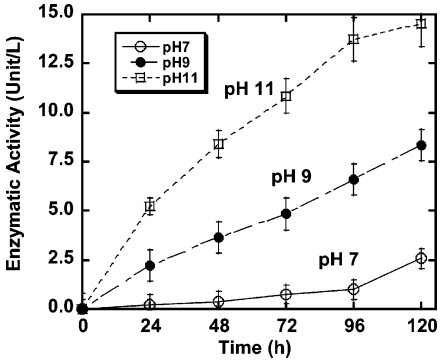

Figure 3. Effect of reaction time on enzymatic activity of DNA−Pt complex at $C_{DNA-Pt} = 50$ pmol/L. DNA−Pt complexes were prepared from $[AG]_{10}$ DNA and $K_2[PtCl_4]$ at pH 7, 9, and 11. Data are expressed as means ± SD of four independent measurements.

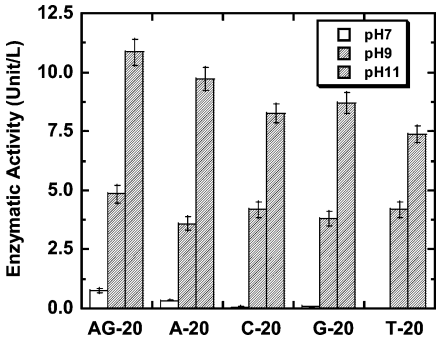

Figure 4. Enzymatic activity of DNA−Pt complex at $C_{DNA-Pt} = 50$ pmol/L. DNA−Pt complexes were prepared from several DNA 20mers at pH 7, 9, and 11 and reaction time of 72 h. Data are expressed as means ± SD of four independent measurements.

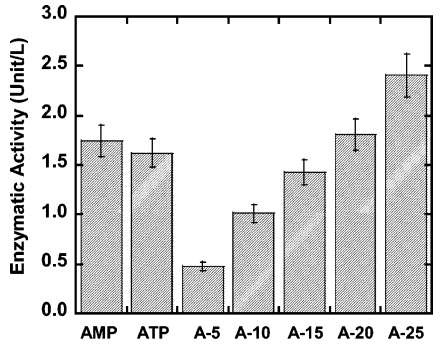

Figure 5. Effect of DNA length on enzymatic activity of DNA−Pt complex at $C_{DNA-Pt} = 50$ pmol/L. DNA−Pt complexes were prepared at pH 9 and 24 h reaction time. Data are expressed as means ± SD of four independent measurements.

$[AG]_{20}$ and A-20mer was found to be higher than for the G-20mer, as shown in Figure 4.

Effect of Oligonucleotide Length on Enzymatic Activity. The enzymatic active site of these complexes was thought to be the platinum bound to the DNA. This would mean that each DNA−Pt complex would have multiple active catalytic sites, whereas a native enzyme such as horseradish peroxidase has only a single active site. The enzymatic activity per unit mole of the DNA−Pt complex was expected to be significantly greater for higher molecular weight DNA. Therefore, the effect of DNA length on the enzymatic activity of these complexes was examined. Figure 5 shows the effect of DNA length on the enzymatic activity of the corresponding complex at 50 pmol/L concentration. The enzymatic activity per unit mole of complex increased with increasing DNA length except for the nucleoside

phosphates AMP and ATP. This was ascribed to the easier binding of platinum to nucleoside phosphates than to DNA oligonucleotides because there was no steric hindrance for the former reaction. The enzymatic activity of the DNA−Pt complex per DNA repeat unit was calculated as 0.096, 0.101, 0.095, 0.090, and 0.096 unit/L for the A-5mer, A-10mer, A-15mer, A-20mer, and A-25mer, respectively. Therefore, the DNA−Pt complex had an average enzymatic activity of 0.096 ± 0.006 unit/L per DNA repeat unit for complexes prepared at pH 9 and 24 h reaction time in this study. This observation also indicated that the active site of the complex was the platinum bound to DNA.

The enzymatic activity of the DNA−Pt complexes prepared from high molecular weight DNA was investigated next. Figure 6 illustrates the enzymatic activity of a DNA−Pt complex prepared with dsDNA from salmon testes at pH 7; with ssDNA from salmon testes at pH 7, 9, and 11; and horseradish peroxidase at the same concentration as that of the DNA−Pt complexes (i.e., 0.064 pmol/L). The DNA−Pt complex prepared from dsDNA showed a weaker enzymatic activity than that containing ssDNA. This was attributed to the double-helical structure of DNA, which prevented the binding of Pt to DNA. This was in keeping with the lack of evidence for DNA being used as a biocatalyst in evolution, as for RNA or proteins. The DNA−Pt complex prepared from ssDNA showed almost the same enzymatic activity as the native HRP enzyme at the same molar concentration.

Calibration Curve of Enzymatic Activity of DNA−Pt Complex and Native Enzyme. HRP has been widely used in ELISA bioassays to detect antigens in a sample (e.g., tumor antigen in blood). In this case, the enzymatic reaction increases when the sample contains a high concentration of antigen because of the increase in conjugation of the antibodies having HRP to the antigen. Thus, ELISA detects the concentration of HRP to quantify the antigen in the sample. One possible application of our DNA−Pt complex is in ELISA assays using the antibody conjugated to a DNA−Pt complex instead of HRP. To test this concept, the DNA−Pt complex and HRP were quantified using their respective enzymatic reactions. Figure 7 illustrates the concentration calibration curves for the complexes and HRP. The equations for the calibration curves using the DNA−Pt complex prepared at pH 7, 9, and 11 and for HRP are as follows:

(a) for the DNA−Pt complex at pH 7

$$\text{absorbance } (-) = 0.083(C_{\text{DNA-Pt}}/[\text{pmol/L}])^{0.754} \quad (2)$$

(b) for the DNA−Pt complex at pH 9

$$\text{absorbance } (-) = 0.482(C_{\text{DNA-Pt}}/[\text{pmol/L}])^{0.758} \quad (3)$$

and (c) for the DNA−Pt complex at pH 11

$$\text{absorbance } (-) = 1.811(C_{\text{DNA-Pt}}/[\text{pmol/L}])^{0.792} \quad (4)$$

where $C_{\text{DNA-Pt}}$ indicates the concentration of the DNA−Pt complex in solution. The molecular weight of HRP was considered to be 40 000 Da for the calculation of the concentration of HRP in a unit of picomoles/liter in Figure 7. The correlation coefficients of the calibration curves using DNA−Pt complexes prepared at pH 7, 9, and 11 were calculated to be 1.000, 0.997, and 1.000, respectively. Thus, there was a good correlation between enzymatic activity and concentration for the DNA−Pt complex.

Thermal Stability of Enzymatic Activity in DNA−Pt Complex and Native Enzyme. One drawback of natural enzymes is their denaturation upon heating. The enzymatic activity of the DNA−Pt complex was compared to that of HRP after solutions of complex and HRP were each heated for 10 min at 20−80 °C, and the results are shown in Figure 8, where the enzymatic activity ratio indicates the ratio of the activity at a certain temperature to the activity at 20 °C. The enzymatic activity of horseradish peroxidase decreased with increasing heat treatment temperature above 60 °C, whereas the activity of the DNA−Pt complex remained constant under the same treatment conditions. Therefore, the DNA−Pt complex was found to have better thermal stability than native HRP.

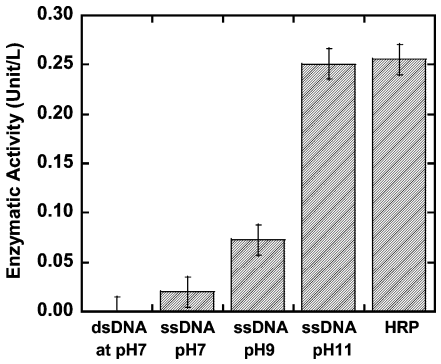

Figure 6. Enzymatic activity at 0.064 pmol/L concentration of DNA−Pt complexes and HRP. DNA−Pt complexes were prepared from dsDNA and ssDNA at pH 7, 9, and 11 and 72 h of reaction time. Data are expressed as means ± SD of four independent measurements.

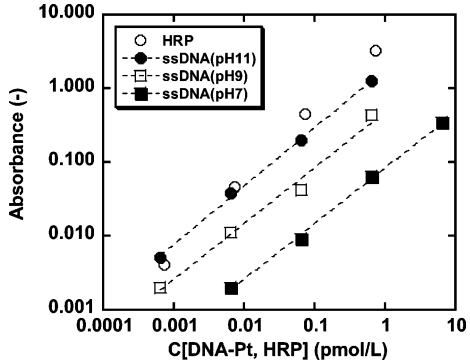

Figure 7. Calibration curve of concentration of DNA−Pt complexes (■, □, and ●) and HRP (○). DNA−Pt complexes were prepared using ssDNA at pH 7 (■), 9 (□), and 11 (●) and 72 h of reaction time.

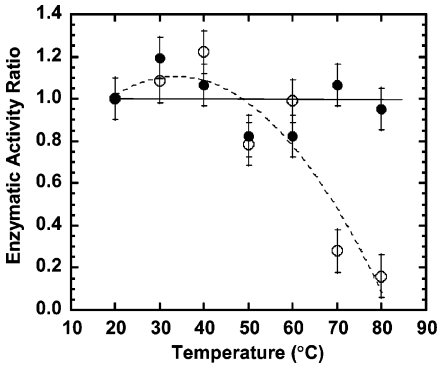

Figure 8. Dependence of enzymatic activity ratio of a DNA−Pt complex (●) and HRP (○) on heat treatment temperature. Both $C_{\text{DNA-Pt}}$ and C_{HRP} were 0.64 pmol/L, and the heat treatments were carried out for 10 min on each sample. The DNA−Pt complex was prepared using ssDNA at pH 9 and 72 h of reaction time. Data are expressed as means ± SD of four independent measurements.

Conclusion

The present study has shown that DNA−Pt complexes possess peroxidase enzymatic activity, even though it has been generally believed that the enzymatic activity of DNA is limited to DNA or RNA cleavage and DNA or RNA modification (such as phosphoesterification). DNA−Pt complexes prepared at a higher pH and with longer reaction times showed a higher peroxidase activity upon the TMB substrate. XPS analysis revealed that this enhanced activity could be attributed to the increase in the amount of Pt conjugated to the DNA in these complexes. The enzymatic activity per unit mole of the DNA−Pt complex was significantly higher for complexes prepared with high molecular weight DNA because their activity per repeat unit of DNA was found to be almost constant when prepared under the same reaction conditions. These complexes showed good thermal stability as compared to the native HRP enzyme. Therefore, such DNA−Pt complexes could be useful as enzymes conjugated to the antibody in ELISA assays due to their high thermal stability, possibly allowing the development of ELISA kits, which do not need refrigeration.

Acknowledgment. This research was partially supported by a Grants-in-Aid for Scientific Research (No. 14655136 and 17560691) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We are grateful for financial support from the Asahi Glass Foundation, the Iketani Science Technology Foundation, and the National Science Council of Taiwan under Grant Nos. NSC95-2221-E-008-086 and NSC96-2218-E-008-007.

Supporting Information Available. Calibration curve of concentration of HRP. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Breaker, R. R. *Nature* **2004**, *432*, 838.
(2) Scherer, L. J.; Rossi, J. J. *Nat. Biotechnol.* **2003**, *21*, 1457.
(3) Kruger, K.; Grabowski, P. J.; Zaug, A. J.; Sands, J.; Gottschling, D. E.; Cech, T. R. *Cell* **1982**, *31*, 147.
(4) Michienzi, A.; Cagnon, L.; Bahner, I.; Rossi, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8955.
(5) Volpe, T. A.; Kidner, C.; Hall, I. M.; Teng, G.; Grewal, S. I.; Martienssen, R. A. *Science* **2002**, *297*, 1833.
(6) Reinhart, B. J; Bartel, D. P. *Science* **2002**, *297*, 1831.
(7) Breaker, R. R. *Science* **2000**, *290*, 2095.
(8) Breaker, R. R. *Nat. Biotechnol.* **1999**, *17*, 422.
(9) Cairns, M. J.; Hopkins, T. M.; Witherington, C.; Wang, L.; Sun, L. Q. *Nat. Biotechnol.* **1999**, *17*, 480.
(10) Santoro, S. W; Joyce, G. F. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4262.
(11) Fahmy, R. G.; Waldman, A.; Zhang, G.; Mitchell, A.; Tedla, N.; Cai, H.; Geczy, C. R.; Chesterman, C. N.; Perry, M.; Khachigian, L. M. *Nat. Biotechnol.* **2006**, *24*, 856.
(12) Liu, Z.; Mei, S. H.; Brennan, J. D.; Li, Y. *J. Am. Chem. Soc.* **2003**, *125*, 7539.
(13) Sreedhara, A.; Li, Y.; Breaker, R. R. *J. Am. Chem. Soc.* **2004**, *126*, 3454.
(14) Sheppard, T. L.; Ordoukhanian, P.; Joyce, G. F. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7802.
(15) Wang, W.; Billen, L. P.; Li, Y. *Chem. Biol.* **2002**, *9*, 507.
(16) Fahmy, R. G.; Dass, C. R.; Sun, L. Q.; Chesterman, C. N.; Khachigian, L. M. *Nat. Med.* **2003**, *9*, 1026.
(17) Mei, S. H.; Liu, Z.; Brennan, J. D.; Li, Y. *J. Am. Chem. Soc.* **2003**, *125*, 412.
(18) Purtha, W. E.; Coppins, R. L.; Smalley, M. K.; Silverman, S. K. *J. Am. Chem. Soc.* **2005**, *127*, 13124.
(19) Vannela, R.; Adriaens, P. *Crit. Rev. Environ. Sci. Technol.* **2006**, *36*, 375.
(20) Tian, Y.; He, Y.; Mao, C. *ChemBioChem* **2006**, *7*, 1862.
(21) Weizmann, Y.; Beissenhirtz, M. K.; Cheglakov, Z.; Nowarski, R.; Kotler, M.; Willner, I. *Angew. Chem., Int. Ed.* **2006**, *45*, 7384.
(22) Ito, Y.; Hasuda, H. *Biotechnol. Bioeng.* **2004**, *86*, 72.
(23) Higuchi, A.; Adachi, S.; Imizu, T.; Ok, Y.-B.; Tsubomura, T.; Hara, M.; Sakai, K. *J. Phys. Chem.* **2000**, *104*, 9864.
(24) Higuchi, A.; Higuchi, Y.; Furuta, K.; Yoon, B.-O.; Hara, M.; Maniwa, S.; Saitoh, M.; Sanui, K. *J. Membr. Sci.* **2003**, *221*, 207.
(25) Matsuoka, Y.; Kanda, N.; Lee, Y. M.; Higuchi, A. *J. Membr. Sci.* **2006**, *280*, 116.
(26) Higuchi, A.; Hashiba, H.; Hayashi, R.; Yoon, B. O.; Hattori, M.; Hara, M. *ACS Symp. Ser.* **2004**, 876.
(27) Higuchi, A.; Sugiyama, K.; Yoon, B. O.; Sakurai, M.; Hara, M., Sumita; M., Sugahara; S., Shirai, T. *Biomaterials* **2003**, *24*, 3235.
(28) Sherman, S. E.; Lippard, S. J. *Chem. Rev.* **1987**, *87*, 1153.
(29) Mansy, S.; Chu, G. Y. H.; Duncan, R. E.; Tobias, R. S. *J. Am. Chem. Soc.* **1978**, *100*, 607.

BM070137I