Medical Chemistry of Polyoxometalates. Part 2*. Enzymatic Study on Binding of Heptamolybdate to DNA

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Polyoxomolybdates have been found to be a new type of antitumor substances. For example, hexakis-(isopropylammonium)heptamolybdate trihydrate $[NH_3Pr^i]_6[Mo_7O_{24}]\cdot 3H_2O$ (PM 8) displayed potent antitumor activity on animal transplantable tumors and human cancer xenograft [1]. In conjunction with the fact that the d^1 configuration of a Mo site in the Mo_7O_{24} framework exhibits a strong cytotoxicity in contrast with the d^0 configuration, the antitumor activity of $[Mo_7O_{24}]^{6-}$ has been proposed to be due to the reduction of $[Mo_7O_{24}]^{6-}$ in tumor cells, which results in repeated cycles of the redox reaction of $[Mo_7O_{24}]^{6-} + e^- + H^+ \rightleftharpoons [HMo_7O_{24}]^{6-}$ in the cells. To try to understand the molecular dynamics of the antitumor activity of PM 8, we have investigated the interaction of PM 8 with DNA by examining effects on the action of several DNA-related enzymes. As described in this letter, both electrophoretic profiles of PM 8-treated pBR322 DNA and the extent of the digestion of several nucleases on the modified DNAs emphasize the non-specific binding of PM 8 single- and double-stranded DNAs with the non-covalent type, which seems to be a factor of the low cytotoxicity of PM 8.

Experimental

PM 8 was synthesized by a published procedure [2]. Plasmid pBR322 DNA was prepared by a standard method for obtaining a negatively supercoiled DNA. Synthetic copolymer poly(dA-dT):poly(dA-dT), salmon sperm DNA, and a transfer RNA mixture were purchased from Pharmacia P-L Biochemicals, Sigma Chemical Co., and Wako Pure Chemical Industries Ltd., respectively. The latter two were purified by an extensive extraction with phenol. Denaturation of DNA was carried out in distilled water at 100 °C for 10 min. All nucleic acids were dissolved in TE buffer (10 mM Tris-HCl (pH 7.5)/0.5 mM EDTA) and stored at 4 °C.

Restriction Endonuclease Digestions

pBR322 DNA (1 μg) was incubated with 2 units of the enzymes (*Dra*I, *Hae*III, *Sau*3AI) at 37 °C for 2 h in the reaction mixture (20 μl) containing 10 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT. All the reactions were stopped by addition of 25 mM EDTA.

S1 Nuclease Digestion

S1 nuclease (Sankyo Chemical Co., Japan) was purified as reported previously [3]. pBR322 DNA (0.5 μg) was incubated with 2.5 units of S1 at 37 °C for 30 min in the reaction buffer (20 μl) containing 45 mM sodium acetate buffer (pH 5.0), 1.5 mM $ZnSO_4$, 1.5 mM $MnCl_2$ and 75 mM NaCl. The reaction was stopped by additions of 100 mM Tris-HCl buffer (pH 8.5) and 10 mM EDTA. One unit of S1 activity is defined as the amount of the enzyme that converts 50% of 0.5 μg single-stranded pBR322 DNA to the acid-soluble form under the above conditions. For large-scale preparation of the S1-generated unit-length linear DNA, pBR322 DNA (20 μg) was digested with 5 units of S1 in 200 μl of the above reaction buffer.

RNA Polymerase Binding

Escherichia coli DNA-dependent RNA polymerase was purchased from Enzo Biochemicals, Inc. The reaction mixture, in a final volume of 20 μl, contained 40 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM DTT, 0.1 mM EDTA, 0.1 mM each of GTP and ATP, 0.5 μg of pBR322 DNA, and 0.2 μg of the RNA polymerase. The incubation was carried out at 37 °C for 15 min.

Bal 31 Nuclease Digestion

*Eco*RI-generated linear pBR322 DNA (2 μg) was incubated with 2.6 units of Bal 31 S-form (Takara Shuzo Co.) at 30 °C for the indicated periods in the reaction buffer (10 μl) containing 20 mM Tris-HCl buffer (pH 7.6), 12 mM $CaCl_2$, 12 mM $MgCl_2$ and 150 mM NaCl. The reactions were stopped by addition of 50 mM EDTA.

T4 DNA Ligase Reactions

T4 DNA ligase was purchased from Takara Shuzo Co. *Eco*RI-generated linear pBR322 DNA (1 μg) was incubated with 3 units of the ligase at 37 °C for 1 h in the reaction buffer (20 μl) containing 66 mM

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Tris-HCl buffer (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT, 0.3 mM ATP and 100 μg/ml bovine serum albumin. The reaction was stopped by addition of 25 mM EDTA.

Agarose Gel Electrophoresis

Agarose slab gels were prepared and DNA samples, after addition of 1/10 volume of 0.05% bromophenol blue–80% glycerol, were electrophoresed in Tris-acetate buffer (40 mM Tris-HCl buffer (pH 8.0)/20 mM sodium acetate/1 mM EDTA). The DNA bands were stained with ethidium bromide (EtBr), visualized using short wavelength UV light and then photographed.

Results and Discussion

Figure 1a shows the agarose gel electrophoretic patterns of the negatively supercoiled pBR322 DNA treated with various amounts of PM 8. Neither the electrophoretic pattern nor the EtBr-fluorescence intensity of DNA depends on the amount of PM 8, indicating no direct scission of the DNA strand by PM 8.

pBR322 DNA treated with PM 8 was digested with restriction endonucleases *Dra*I, *Hae*III and *Sau*3AI and then electrophoresed. Results are shown in Fig. 1b, where PM 8 is shown to inhibit the pBR322 cleavage induced by the three restriction endonucleases and the degree of the inhibition increases with increasing amounts of PM 8. In addition, the degree of the inhibition by PM 8 decreased with increasing amounts of DNA (data not shown). Thus, the inhibition by PM 8 can be ascribed to binding of PM 8 to DNA rather than to enzyme proteins. Since base sequences and numbers of the recognition-cleavage sites are TTT↓AAA and 3 for *Dra*I, GG↓CC and 22 for *Hae*III, and ↓GATC and 22 for *Sau*3AI, the almost perfect protection of DNA by PM 8 [of e.g. 25 μg/ml against *Hae*III (lane 8 in Fig. 1b)] in contrast to the partial protection against *Sau*3AI (lane 14 in Fig. 1b), implies that PM 8 possesses a weak preference for G and/or C.

The hypothesis of the binding of PM 8 to DNA is supported by an increase in the rate of the *Dra*I-digestion of pBR322 in the coexistence of double- and single-stranded DNAs, as shown in Fig. 2. When pBR322 DNA was incubated with constant concentrations of PM 8 and *Dra*I in the presence of synthetic poly(dA-dT):poly(dA-dT) or heat-denatured salmon sperm DNA as a competitor, the rate of *Dra*I-digestion of pBR322 increased due to competitive binding of PM 8 to the DNAs. On the other hand, the coexistence of tRNA displays no observable increase in the rate of *Dra*I-digestion of pBR322, irrespective of high concentration of tRNA (Fig. 2), suggesting that PM 8 does not have any affinity for RNA. Furthermore, when pBR322 DNA was added to the mixture of PM 8 and *E.coli* RNA polymerase, the gel electrophoretic mobility of pBR322 DNA increased with an increase in the concentration of PM 8. This can be explained in terms of the preferential binding of PM 8 to DNA, which results in the inhibition of the binding of RNA polymerase to DNA. Similarly, the binding of PM 8 depressed Bal 31 exonuclease

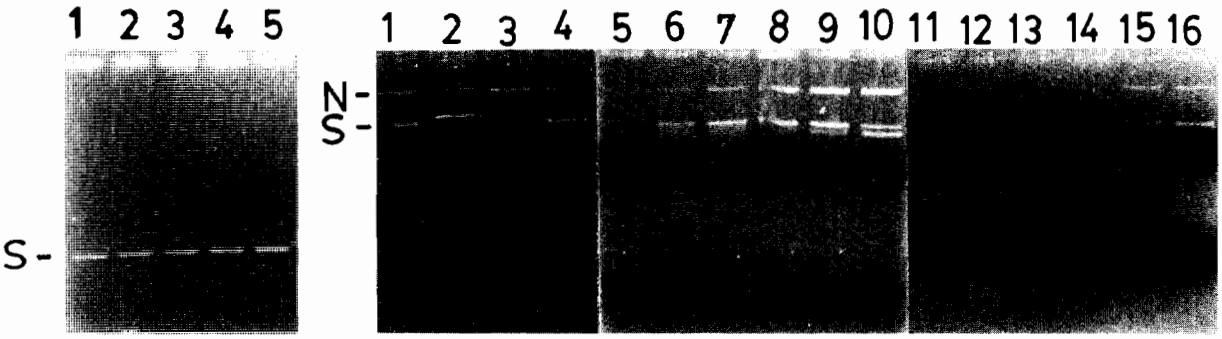

Fig. 1. Agarose gel electrophoretic patterns of pBR322 DNA treated with PM 8 (a); and the restriction endonuclease-digests of PM 8-treated pBR322 DNA (b). (a) pBR322 DNA (1 μg) was incubated at 37 °C for 15 min in TE buffer (20 μl) with the following concentrations of PM 8: lane 1, 0; 2, 10; 3, 25; 4, 50; 5, 100 μg/ml. Electrophoresis was performed in 1% agarose gel at 3 V/cm for 3 h. (b) pBR322 DNA (1 μg) was incubated at 37 °C for 15 min in the restriction endonuclease reaction buffer (20 μl) with the following concentrations of PM 8: lanes 1, 2, 5 and 11, 0; lanes 6 and 12, 5; lanes 7 and 13, 10; lanes 8 and 14, 25; lanes 3, 9 and 15, 50; lanes 4, 10 and 16, 100 μg/ml. The reaction mixtures were treated without the enzyme (lane 1) and with *Dra*I (lanes 2–4), *Hae*III (lanes 5–10) and *Sau*3AI (lanes 11–16). Electrophoresis was carried out in 1% agarose gel at 2.5 V/cm for 1 h. Capital letters N and S are nicked and supercoiled forms of pBR322, respectively.

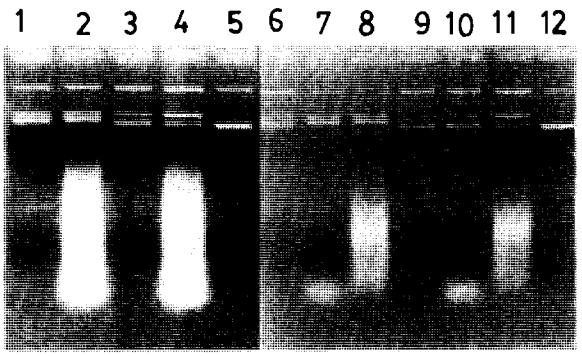

Fig. 2. Influences of nucleic acids on the degree of pBR322 *Dra*I-digestion in the absence or presence of PM 8. pBR322 DNA (1 μg) and the following nucleic acids (1 μg each) were incubated in the *Dra*I reaction buffer (20 μl) at 37 °C for 15 min without (lanes 1, 2, 6, 7 and 8) and with 35 μg/ml PM 8 (lanes 3, 4, 9, 10 and 11) and then digested with *Dra*I. pBR322 DNA alone (lanes 5 and 12) was also incubated. Lanes 2 and 4, poly(dA-dT):poly(dA-dT); lanes 7 and 10, tRNA; lanes 8 and 11, heat-denatured salmon sperm DNA. Electrophoresis was carried out as in the legend to Fig. 1b.

activity which degrades both the 3′- and 5′-termini of the linear duplex DNA produced by *Eco*RI digestion of pBR322. The T4 DNA ligase-catalyzed end-to-end joining of duplex DNA was inhibited under high concentration (250 μg/ml) of PM 8.

The binding of PM 8 to DNA induced no change in the electrophoretic mobility (Fig. 1a). This let us assume that the PM 8 binding to DNA is so non-covalent that PM 8 is dissociated from DNA during gel electrophoresis. The negative supercoiling in DNA introduces localization of the unwinding of helical base-pairs [4]. Since S1 endonuclease exhibits a specificity for digesting the single-stranded region in DNA, the locally denatured region is recognized and cut by S1 nuclease, although single base-pair mismatches are not recognized [4, 5]. *cis*-Diammine-dichloroplatinum(II) (*cis*-DDP) unwinds the double helix when it binds to DNA through covalent attachment to the N7 atoms of guanine. Such perturbations in structure produced by *cis*-DDP stimulate S1 digestion greatly [6]. In contrast with *cis*-DDP, however, PM 8 hardly affected the S1-susceptibility of supercoiled pBR322 DNA.

As shown by the above results, nuclease probes which are sensitive to modifications of DNA clearly indicate that $[Mo_7O_{24}]^{6-}$ binds to double- and single-stranded DNAs with little recognizable modifications of DNA structure. The difference in the mode of binding between PM 8 and *cis*-DDP implies that the binding of the $[Mo_7O_{24}]^{6-}$ anions induces no significant intrastrand crosslinks to DNA bases, in contrast to the most likely structure for the cytotoxic *cis*-DDP–DNA linkage with the covalent attachment of $[Pt(NH_3)_2Cl]^+$ cations produced by the dissociation of Cl^- to the N7 atoms of adjacent guanine bases (for a review, see ref. 7). Combined with the ease of the intercalation of square-planar *cis*-DDP into DNAs, therefore, the non-specific binding of PM 8 to DNA could occur predominantly on the surface of DNAs, since the possibility of the intercalation of non-planar PM 8 into DNA is unlikely. Thus, the non-specific and probably non-covalent binding of PM 8 to DNA may be directly related to its low cytotoxicity. A more detailed analysis is required to elucidate the mechanism of the PM 8 binding.

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