J. Biochem. Mol. Biol. Vol. 28, No. 1 pp. 1~5 Januray 31, 1995

Structural Damage of DNA by 6-Sulfooxymethyl Benzo(a)pyrene

Young Sik Cho and An-Sik Chung

Department of Life Science, KAIST, Taejon 305-338, Korea

(Received July 7, 1994)

Abstract: The effect of 6-sulfooxymethyl benzo(a)pyrene (SMBP) on conformational changes of calf thymus DNA was investigated. As SMBP is a strong electrophile, the covalent binding of SMBP to DNA should distort three dimensional conformation of DNA at the binding sites. A formaldehyde-unwinding methods were used to determine the rate of DNA denaturation. The increase in absorbance at 251nm was detected by addition of formaldehyde following treatment with SMBP. SMBP changed supercoiled DNA to relaxed and linear DNA as determined by electrophoresis, which was similar to the change in DNA due to *in vitro* treatment with benzo(a) pyrene diol epoxide.Treatment with SMBP completely denatured DNA under alkaline conditions. However, DNA was nicked or partially denatured under neutral condition. The absorption band of DNA was increased by the treatment with SMBP in V79 cells, which may be explained by the formation of stabilized SMBP-DNA adduct.

Key words: Conformation, DNA, benzo(a)pyrene, sulfoester.

Exposure to various chemicals, either due to occupation or lifestyle, is considered as a major contributing factor to tumor induction in man (Higginson, 1969; Doll *et al.*, 1981). An important and prevalent class of potent carcinogenic compounds found in the environment is the polycyclic aromatic hydrocarbons (PAHs), which are present in various petroleum and are liberated as incomplete combustion products derived from heat and power generation, and as motor vehicle exhausts. Benzo(a)pyrene (B(a)P) has been studied by various groups and the toxic biochemical mechanisms of it have been known to be ascribed to the diol-epoxide formed by cytochrome P450 in the liver. But recently, it was proposed that some forms of PAH have toxic effects on cells through another metabolic pathway. PAHs containing meso-methyl group are supposed to be hydroxylated by cytochrome P450, NADPH and O_2 in the microsomes, and subsequently form reactive benzylic esters bearing leaving groups (e.g., sulfate, phosphate and acetate) in the cytosol (Flesher *et al.*, 1973). Metabolic activation of methyl-substituted PAH is shown in Fig. 1. SMBP derived from a mechanism mediated by sulfotransferase forms the electrophile and this electrophile nonenzymatically forms adduct with macromolecules such as DNA (Flesher *et al.*, 1971) and protein (Watabe *et al.*, 1983). Formation of sulfoesters of some hydroxymethyl PAHs *in vitro* by rat hepatic sulfotransferase activity has been suggested as support for this idea (Watabe *et al.*, 1982). Sulfotransferase-mediated activation of proximate carcinogens was firstly proposed by Miller and Miller (1975) with N-hydroxy-2-acetylaminofluorene and N-hydroxy-methylaminobenzene, a putative oxidative metabolite of methyl aminobenzene. To understand carcinogenic mechanism at the molecular level, it is necessary to understand

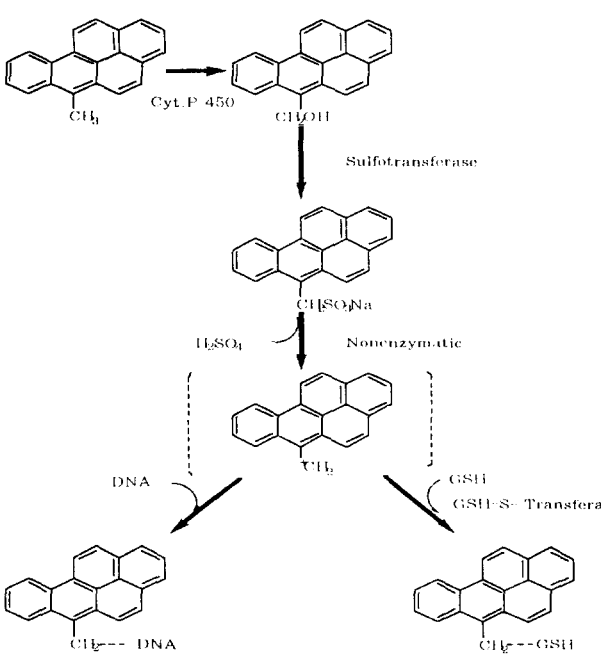

Fig. 1. Hydroxylation-esterification pathway for the metabolic activation of methyl substituted polycyclic aromatic hydrocarbon to form benzylic DNA adducts (Surh *et al.*, 1989a).

the complete chemical structure and stereochemistry of the carcinogen-macromolecule adduct, the associated conformational change in the target macromolecules, and the relationship between chemical or physical factors inducing cancer and the possible aberrations in the functional properties of these macromolecules. The purpose of this study was to elucidate the three dimensional conformation of the double helical DNA at modification sites and further to investigate the relationship between conformation and macroscopic cellular aberration.

Materials and Methods

Materials

Calf thymus DNA, agarose and ultrapure cesium sulfate were purchased from Sigma Co., Ltd. (St. Louis, USA). Formaldehyde was obtained from Junsei Co., Ltd. (Tokyo, Japan). Restriction enzymes to confirm the conformation of pUC 9 were obtained from Boehringer Mannheim Co., Ltd. (Mannheim, Germany). SMBP was obtained from Dr. Y. J. Surh at Yale University, USA and pUC 9 was purified from *E. coli*.

Methods

Formaldehyde-unwinding Method: The formaldehyde -unwinding method (Utiyama *et al.*, 1974) was used to assess the extent of denaturation as SMBP was added to DNA. SMBP-treated calf thymus DNA was incubated in reaction mixture containing 0.042 M sodium borate (pH 9.0) and 1 M formaldehyde, and compared to native and heat denatured DNA. The kinetics of formation of formaldehyde adducts with DNA were monitored using a DU-7 UV-visible spectrophotometer. The wavelength generally used was 251 nm, which represent an isobestic point for adduct formation in the absence of changes in the extent of base-stacking (von Hippel *et al.*, 1971).

Centrifugation :

(a) Sucrose gradient centrifugation under neutral condition

An aliquot (0.1 ml) of DNA (1.0 mg/ml) was applied to the top of a 10~30% sucrose gradient in 0.01 M sodium cacodylate (pH 7.1), 0.1 M NaCl and 1×10^{-3} M EDTA. The gradient samples were centrifuged in Beckman 80 Ti rotor at 45000 rpm for 300 min at 20℃.

(b) Sucrose gradient centrifugation under alkaline condition

An aliquot (0.1 ml) of DNA sample in 0.2 M NaOH was applied to the top of 5~20% sucrose gradient in 0.1 M NaOH and 0.9 M NaCl. Gradients were centrifuged in Beckman 80 Ti rotor at 45,000 rpm for 240 min at 20℃. Fractions were collected from the bottom of the tubes and was measured using a DU-7 spectrophotometer at 260 nm .

(c) Cesium sulfate ultracentrifugation

This procedure was performed by the previously described method (Michael *et al.*, 1979). Chinese hamster lung fibroblast (V79) Cell lines were harvested by treatment for 15 min at 37℃ with 0.05% trypsin, 0.02% EDTA, and washed in RSB (10 mM NaCl, 10 mM Tris (pH 7.0), 1.5 mM $MgCl_2$). V79 Cells were suspended in RSB and lysed by potter-Elvehjem homogenization after the addition of 0.5% Triton X-100 and 0.5% sodium deoxycholate. Nuclei were pelleted by low-speed centrifugation. Nuclear macromolecules obtained from SMBP treated V79 cells were dissolved in 6M guanidine-HCl. Aliquots (2.2 ml) of the nuclear preparation were layered on 2.8 ml of a solution containing 2.2 M Cs_2SO_4, 10 mM EDTA (pH 7.0), and 9% DMSO and centrifuged for 40 h in a SW55 Ti rotor at 35000 rpm at 20℃. Tubes were pierced near the bottom and 5- or 6-drop fractions were collected. Aliquots (20 μl) of samples were diluted to 1.0 ml by deionized water. Nucleic acids were determined by measuring the absor-

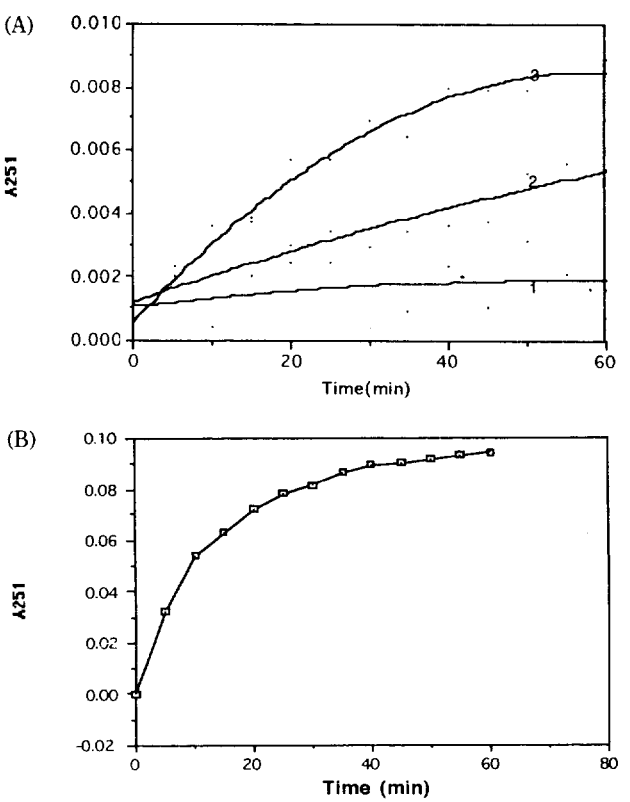

Fig. 2. Kinetics of formaldehyde unwinding of DNA samples. Increase in optical density of DNA at 251 nm was followed in reaction mixtures containing 0.042 M sodium borate (pH 9.0), 1 M formaldehyde ; A: (1) native DNA; (2) 1 nmol SMBP modified DNA; (3) 5 nmol SMBP modified DNA, B: Heat denatured DNA.

bance at 260 nm and protein was assayed by fluorescamine method (Udenfried *et al.*, 1972).

Electrophoresis : pUC 9 plasmids were incubated in several concentrations of SMBP (0, 0.05, 0.5, 5 nmol) for 30 min at 37℃, followed by electrophoresis in 0.8% agarose at 100 V for 60 min. The gel was stained with EtBr and the fluorescent DNA bands were photographed.

Results and Discussions

The formaldehyde was used as a chemical prove to measure the number of open base pairs in double helical DNA at temperature below Tm because the formaldehyde reacts preferentially with bases in the open regions of helical structure. The effect of SMBP on binding of formaldehyde to calf thymus DNA was shown in Fig. 2. The treatment with 1 nmol SMBP significatly increased the absorbance at 251 nm compared with that of native DNA and further 5 nmol SMBP showed a dramatic increase in absorption, which was similar trend to that of heat denatured DNA but not quite at the same level. These results suggest that SMBP could increase the formaldehyde reactivity to bases in the open region of double helical DNA. The initial rate constants of unwinding for the native, heat denatured, SMBP treated calf thymus DNA were shown in Table 1. Treatmen with 1 nmol SMBP raised the relative fraction of open base pairs from 5 base pairs of control to 40.5 base pairs and treatment with 5 nmol SMBP resulted in a level of three times higher than that of 1 nmol SMBP treated DNA. Surh *et al.* (1989b) demonstrate that sulfotransferase activity for 6-hydroxymethyl benzo(a)pyrene (HMBP) in rat and mouse liver cytosols is mediated to form benzylic adducts from hydrocarbons with guanosine and deoxyguanosine. Furthermore, these benzylic adducts are also obtained from reaction of synthetic SMBP with individual (deoxy) ribonucleotides or DNA and the major DNA adduct formed with SMBP was proved to N^2-(benzo[a]pyrene-6-methylenyl)-deoxyguanosine. This study suggests that the treatment with SMBP on the calf thymus DNA produce large regions of open base pairs. The SMBP intercalates and binds with DNA double helical regions, in which open base pairs occur. Binding studies of diol epoxide of BP to DNA was conducted by Bannon *et al.* (1972). The reaction of diol epoxides with 5′-GMP suggests that the hydrocarbons react with the phosphodiester backbone of DNA and RNA to give

Table 1. Calculation of relative fraction of open base introduced by SMBP bound to calf thymus DNA

DNA	$K_0 10^4$/min[a]	$\theta_n(T)$[b]	$\theta_{SMBP}(T)$[c]
Heat-denature	5410		
Native DNA	5	0.0009	
1 nmol SMBP-modified	40.5		0.0075
5 nmol SMBP-modified	131		0.024

[a]Initial rate constant.
[b]The relative fraction of open base pairs at a given temperature.
[c]The ralative fraction of open base pairs in the SMBP treated samples.

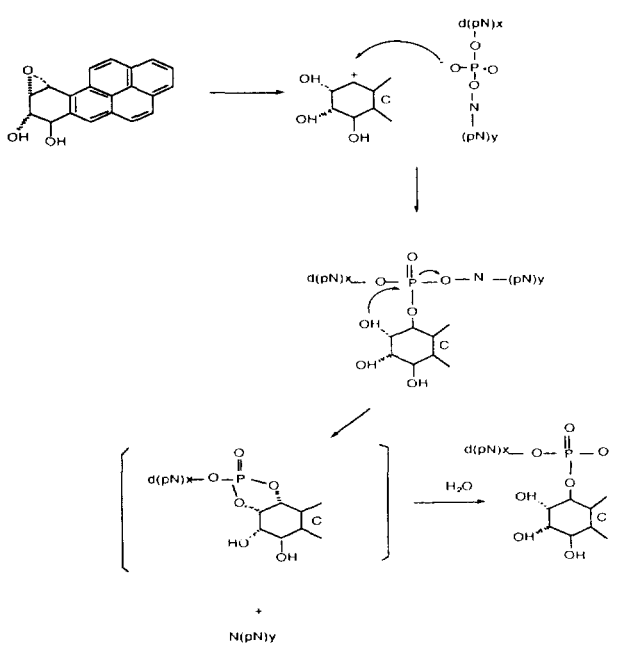

Fig. 3. Postulated mechanism of DNA strand scission by BP diol epoxide (Rhaese *et al.*, 1969).

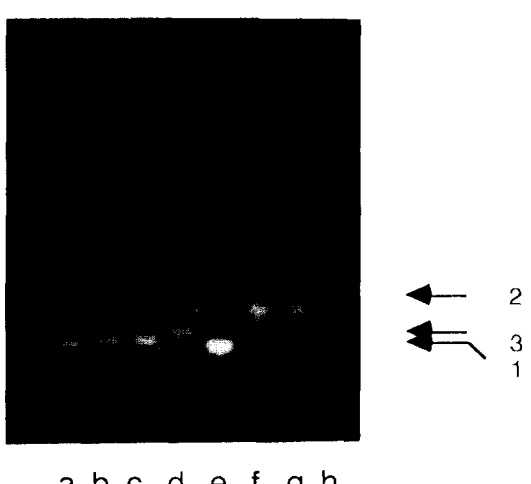

Fig. 4. Agarose gel electrophoresis (0.85%) of pUC 9 treated with SMBP. Aliquots of 15 μl aliquots were electrophoresed at 100 V for 60 min. Mark 1, 2, 3 correspond to superhelical, relaxed and linear pUC 9 plasmid DNA, respectively. Plasmid was incubated at 37℃ with , (a) 0, (b) 0.05, (c) 0.1 nmol, (d) 0.25 nmol SMBP, (e) DMSO, (f) 0.5 nmol, (g) 1 nmol and (h) 5 nmol SMBP.

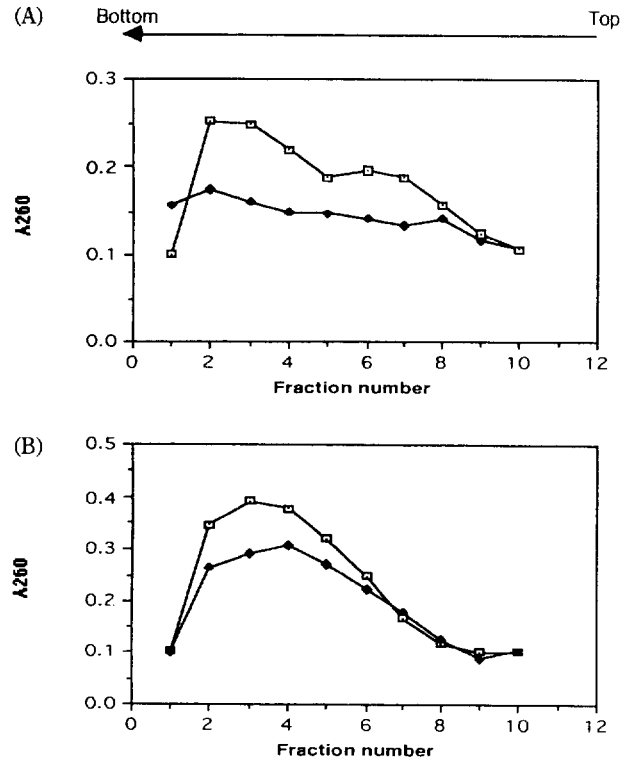

Fig. 5. Sucrose gradient centrifugation of DNA. (A) and (B) represent alkali and neutral gradients, respectively. Detail procedures are described under Materials and Methods. □—□: native DNA; ■—■: 100 μM SMBP-treated DNA.

unstable phosphotriesters and that subsequent triester hydrolysis could lead to strand scission.

A postulated mechanism of DNA strand scission by BP diol epoxide is shown in Fig. 3. This is a two step process consisting of loss of an alkylated base followed by β-elimination. The β-hydroxyl group of the hydrocarbon mediated unstable phosphotriesters can be rapidly hydrolyzed to cause scission of RNA (or DNA) strand. The above mechanism of RNA strand by BP diol epoxide can be applied to a similar mechanism of strand scission by SMBP. Generally, the scission of DNA strands occurs through depurination or depyrimidination (Singer *et al.*, 1975). As shown in the structure of SMBP, there are no hydroxyl groups to react with the amino groups of guanosine and adenine residues, and these adducts can be rapidly cleaved to produce an apurinic or apyrimidic sites. The scission mechanism of DNA strands is derived from the slow process of β-elimination under physiological condition (Rhase *et al.*, 1969; Straus *et al.*, 1968). To investigate this possibility, superhelical pUC 9 DNA was reacted with various concentrations of SMBP and then subjected to agarose gel electrophoresis. Electrophoresis results demonstrated that an increasing concentration of SMBP first nicked and then broke DNA finally (Fig. 4). Similar results were shown with BP diolepoxide treated DNA.

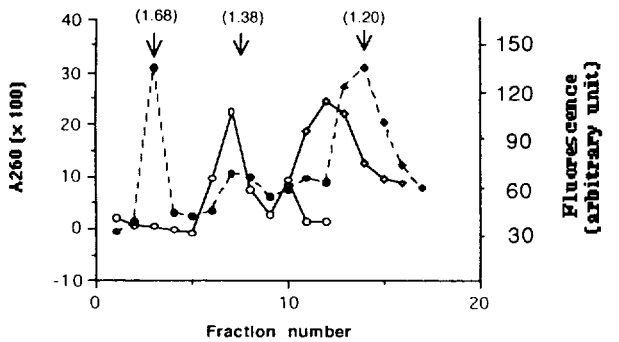

Fig. 6. Isopycnic separation of nuclear macromolecules by the treatment of 4 μM SMBP per plate of V79 cells, Nuclei from SMBP-treated V79 cells were subjected to isopycnic centrifugation and analyzed as described under Materials and Methods. Arrows above panel represent the density of fraction. Density bands of 1.68, 1.38 and 1.2 (g/cm^3) mean DNA, RNA and protein respectively. Untreated Nuclei; ●—●: A_{260}, Treated Nuclei; ○—○: A_{260}, ■—■: Fluorescence, □—□: Fluorescence.

Most of pUC 9 represented supercoiled DNA and only small fraction of relaxed DNA. DMSO and 0.1 nmol SMBP-treated pUC 9 was not affected. However, most of superhelixed DNA was shifted to relaxed DNA in 0.5 nmol SMBP-treated pUC 9, and treatment of 2 nmol SMBP cleaved the pUC 9 DNA to form linear DNA.

The absorbance band of calf thymus DNA treated with SMBP disappeared (Fig. 5). This indicated that nicked double strands were cleaved to small single strand fragments under alkaline condition. The sucrose gradient profile of SMBP treated DNA showed a curve similar to control DNA under neutral condition, which indicates that they are nicked double strand or partial cleavage products of the DNA. Isopycnic separations of RNA, DNA, and proteins from SMBP-treated V79 cell were shown in Fig. 6. The RNA fraction was not detected in SMBP-treated sample, in which RNA was cleaved with β-elimination by SMBP. However, treatment with SMBP increased the absorbance of DNA fraction, which may be explained by stabilized SMBP -DNA adduct. The protein band was shifted to higher buoyant density compared with that of control. This reacts may be derived from SMBP-protein adducts. In the case of 7-hydroxymethyl-12-methyl benzo(a)anthracene (HMBA), this proximate carcinogen binds covalently to liver cytosolic protein via 7-HMBA sulfate (Watabe *et al.*, 1983). Further studies of circular dichroism and NMR on the SMBP-DNA and the SMBP-protein interaction will clarify the change of DNA structure.

Aknowledgement

This study was supported by grant 921-0400-02202 from Korea Science and Engineering Foundation.

References

Bannon, P. and Verly, W. (1972) *Eur. J. Biochem.* **31**, 103.

Doll, R. and Peto, R. (1981) *J. Natn. Cancer Inst.* **66**, 1191.

Flesher, J. W. and Sydnor, K. L. (1971) *Cancer Res.* **31**, 1951.

Flesher, J. W. and Sydnor, K. L. (1973) *Int. J. Cancer Res.* **11**, 433.

Higginson, I. (1969) *Can. Cancer Confor.* **8**, 40.

Michael, C. M., Betty, K. M., Anne, H. and James, K. S. (1979) *Anal. Chem.* **97**, 410.

Miller, J. A. and Miller, E. C. (1975) *Biochemical Reactive Intermediates*, p. 6. Plenum Press, New York.

Rhase, H. J. and Freese, E. (1969) *Biochem. Biophys. Acta.* **190**, 418.

Singer, B. (1975) *Prog. Nucleic Acid Res. Mol. Biol.* **15**, 219.

Strauss, B., Coyle, M. and Robbins, M. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33**, 277.

Surh, Y. J., LAi, C. C., Miller, J. A. and Miller, E. C. (1989a) *Proc. Am. Assoc. Cancer Res.* **30**, 121.

Surh, Y. J., Liem, A., Miller, E. C. and Miller, J. A. (1989b) *Carcinogenesis* **10**, 1519.

Udenfried, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W. and Wiegele, M. (1972) *Science*, **178**, 871.

Utiyama, H. and Doty, P. (1974) *Biochemistry* **10**, 1254.

von Hippel, P. H. and Wong, K-Y. (1971) *J. Mol. Biol.* **61**, 587.

Watabe, T., Ishizuka, T., Hakmato, Y., Atzawa, T. and Isobe, M. (1983) *Biochemical Phamacology* **32**, 2120.

Watabe, T., Ishizuka, T., Isobe, M. and Ozawa, N. (1982) *Science*, **215**, 403.