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cis-Diamminedichloroplatinum (II) induces denaturation and conformational changes in pBR322 DNA

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Abstract : *E. coli* LE392, transformed with CDDP-treated pBR322 DNA, was plated on ampicillin containing media. The number of colonies formed on ampicillin containing agar plate was reduced to undetectable level after treat the DNA with 13.3 μM CDDP. The CDDP-treated pBR322 DNA was susceptible to single strand DNA specific S1 nuclease and it's migration pattern in agarose gel electrophoresis was changed. These results suggest that CDDP adduction to pBR322 DNA resulted in denaturation of the double helix and changes in it's conformation which ultimately leads to the inactivation of the ampicillin resistant gene(Received November 25, 1990, Accepted December 22, 1990).

cis-Diamminedichloroplatinum(II)(CDDP) has been established as an antitumor drug, whereas its trans isomer exhibits little antitumor activity(Roberts and Thomson, 1979). In aqueous solution, both chloride groups of CDDP are slowly displaced by water molecules thus generating a distribution of unhydrolyzed and of partially and fully hydrolyzed species(Lippard, 1982).

The molecular mode of action of the drug is not clear but it is widely believed that the primary intracellular binding site is DNA(Roberts and Thomson, 1979). Mansy et al.(1973) reported that CDDP bound to adenosine, cytidine and guanosine but not to uridine and thymidine. The requirement of cis conformation for antitumor activity leads to initial suggestion that CDDP formed crosslinks between DNA strands and on the same strand through the bifunctional coordination. In addition to intrastrand(Marcelis et al., 1983 ; Malinge and Leng, 1984 ; Fichtinger-Schepman et al., 1985) and interstrand(Zwelling et al., 1978) crosslinks, monoadduct formation was also reported(Fichtinger-Schepman et al., 1985).

Among number of possible modes of action of CDDP, including inhibition of protein and RNA synthesis, numerous studies have indicated that CDDP exercises it's antitumor activity by inhibiting cellular DNA replication (reviewed by Roberts and Thomson, 1979). The inhibition of DNA replication was presumed to be due to the binding of platinum complexes to the DNA template and interference with chain elongation.

The mode of action of CDDP on pBR322 DNA was investigated. The alterations in DNA conformation due to the CDDP adduction were investigated by analysing the changes in migration patterns in agarose gel electrophoresis and susceptibility to single strand DNA specific nuclease S1.

Materials and Methods

Cell and media

E. coli LE392 grown in LB broth(10g bacto trypton, 5g yeast extract, and 10g NaCl per liter) was used for the propagation of pBR322 DNA and for the transformation. Transformed cells were grown in LB agar plate containing 100μg/ml of ampicillin at 37℃.

DNA preparation

pBR322 DNA was isolated from *E. coli* LE392 as described in Birnboim(1983). Form III DNA was prepared by digesting form I DNA with Hind III. Lambda DNA was purchased from Promega Biotech.

CDDP(or TDDP) reaction with pBR322 DNA

Key words : cis-Diamminedichloroplatinum(II), pBR332, form I DNA, conformation, S1 nuclease
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CDDP(Sigma) stock solution was prepared by dissolving CDDP either in 20mM KH_2PO_4 buffer, pH 7.2, (6mg/ml) or double deionized H_2O by heating in a boiling water bath for 5min in the dark. Only freshly prepared CDDP (less than 1hour old) was used. To purified pBR322 DNA, previously diluted into 1 : 4 with 20mM KH_2PO_4 (pH 7.2), an equal volume of CDDP of twice the final concentration was added. The reaction mixture was incubated for 1hr at 37℃ in the dark and the reaction was stopped by adding either 1X SSC(0.15M NaCl, 0.015M Na-citrate, pH 7.2) or electrophoresis loading buffer.

Transformation

Transformation was done as described in Cohen et al. (1972). For the preparation of competence cell, *E. coli* LE392 was cultured in LB broth with vigorous shaking until it's O. D. at 550nm reached to 0.5. The cells, pelleted by centrifugation at 10,000Xg for 5min, were washed twice with half and one-tenth volumes of ice cold transformation buffer(10mM Tris-HCl, pH 7.5 and 50mM $CaCl_2$) and stored at 4℃. One μg of pBR322 DNA mixed with 0.2ml of competent cells of LE392 was incubated for 30min at 4℃ followed by 2min heat shock at 42℃. After chilling on ice water bath, 1ml of LB broth was added and incubated for 1hr at 37℃ for phenotypic expression. The transformants were plated out on LB ampicillin plate.

Agarose gel electrophoresis

Agarose gels prepared in TEA buffer(0.04M Tris-acetate, 2mM EDTA, pH 8.0) were run in horizontal submarine gel apparatus at 5V/cm for 6hr. Gels were stained with ethidium bromide(EtBr)(0.5μg/ml) for 40min and visualized using a UV lamp.

S1 nuclease digestion of CDDP-treated DNA

CDDP-treated pBR322 DNA was digested with 0.5unit of S1 nuclease(Sigma) in 50mM sodium acetate (pH 4.6), 200mM NaCl, and 1mM $ZnSO_4$ at 37℃ for 30min. The reaction was stopped by adding 0.5M EDTA.

Results and Discussion

Transformation of CDDP-treated pBR322 DNA

Given the known reactivity of CDDP with nucleic acid together with it's ability to reduce gene activity, experiments were conducted to determine the degree of effectiveness of CDDP on the inactivation of β-lactamase gene on pBR322. pBR322 DNAs exposed to various concetrations of CDDP for 60min at 37℃ in the dark, were transformed into competent cells of *E. coli* LE392 and the tranformed cells were plated on LB-ampicillin agar plate.

As can be seen in Figure 1, a concentration of as little as 3.3μM CDDP led to a substantial reduction in the number of viable colonies on the LB-ampicillin plate. When pBR322 DNA was exposed to 13.3μM CDDP, the number of colonies formed on LB-ampicillin plate was totally reduced to undetectable level. This result agrees with the previous report that CDDP effectively inactivates gene activity. On the other hand, 133.3μM TDDP, approximately ten times more concentrated than CDDP, was required to have comparable effect(Fig. 1). These results are consistent with the previous observation that TDDP is less effective than CDDP in it's binding affinity to DNA and in its antitumor activity.

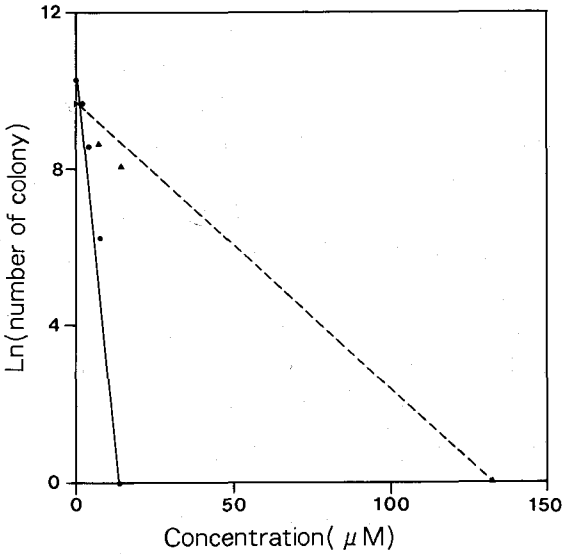

Fig. 1. Number of viable colonies formed on ampicillin containing LB plate.

Competent cells of *E. coli* LE392, transformed with CDDP-treated pBR322 DNA, were plated on LB-ampicillin agar. Number of colonies were counted after 1~2 days of incubation at 37℃. ●—● ; CDDP, ▲—▲ ; TDDP.

Agarose gel electrophoresis of CDDP-treated form I pBR322 DNA

To investigate alterations in DNA conformation by CDDP adduction, form I pBR322 DNAs treated with increasing concentrations of CDDP were electrophoresed in 1% agarose gel. Figure 2 shows that DNA migrated slower as CDDP concentration increases, then migrated faster when CDDP concentration increased further. This result is analogous to the observation that was made with form I DNA treated with EtBr.

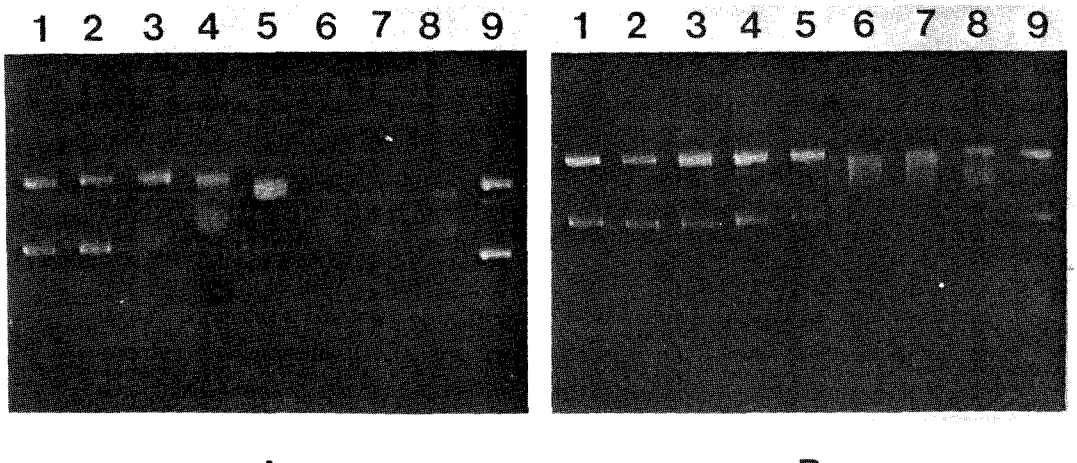

Fig. 2. Electrophoresis of CDDP(or TDDP)-treated form I pBR322 DNA in 1% agarose gel.

Electrophoresis was performed in horizontal submarine gel at 5V/cm for 6hr. The DNA bands were stained with 0.5μg/ml of EtBr for 30min. Lane 1 and 9 ; control, lane 2; 10μM CDDP(TDDP), lane 3; 25μM CDDP(TDDP), lane 4 ; 50μM CDDP(TDDP), lane 5; 100μM CDDP(TDDP), lane 6 ;200μM CDDP(TDDP), lane 7 ; 500μM CDDP(TDDP), lane 8; 1mM CDDP(TDDP). Panel A ; CDDP, panel B ; TDDP.

The observed migration pattern could be interpreted as that CDDP unwinds and rewinds the form I DNA. The unwinding of negative supercoiled DNA results in retardation of mobility in agarose gel(Fig. 2, panel A, lane 2, 3 and 4). At the CDDP concentration where the negative supercoil was completely unwound, the conformation would be changed into relaxed form whose mobility is maximally retarded(Fig. 2, panel A, lane 5). Futher adduction of CDDP on DNA would change it's conformation into positively supercoiled form resulting in enhancement of mobility in agrose gel(Fig. 2, panel A, lane 6, 7 and 8). TDDP treated form I DNA exhibited similar migration pattern although it's mobility was not changed as much that of CDDP treated form I DNA.

Agarose gel electrophoresis of CDDP-treated form pBR322 DNA

Mobility of nucleic acid in agarose gel depends on several factors. Given the same molecular weight, charge distribution, ionic strength in the buffer, and voltage gradient, the mobility of nucleic acids largely depends on it's conformation(Johnson and Grossman, 1977). It has been reported that psoralen, a cross-linking agent, adduction to DNA generates conformational changes by bending the DNA. If CDDP adduction to DNA changes its conformation into compact and short form by bending the DNA, then the CDDP-treated DNA move faster than control DNA because mobility in the agarose gel is inversely proportional to its length(Lerman and Frisch, 1982 ; Lumpkin and Zimm, 1982).

Form Ⅲ DNAs treated with increasing concentrations of CDDP, were electrophoresed in 1% agarose gel(Fig. 3, panel A). As CDDP concentration increased, DNA migrated faster in 1% agarose gel(Fig. 3, panel A, lane 2 to 7). This result suggests that CDDP adduction to DNA induces changes in it's conformation into compact form. TDDP-treated DNA showed little change on the mobility in agarose gel(Fig. 3, panel B).

Cruciform DNA was shown to exhibit unusual gel electrophoretic mobility due to the introduction of a bend

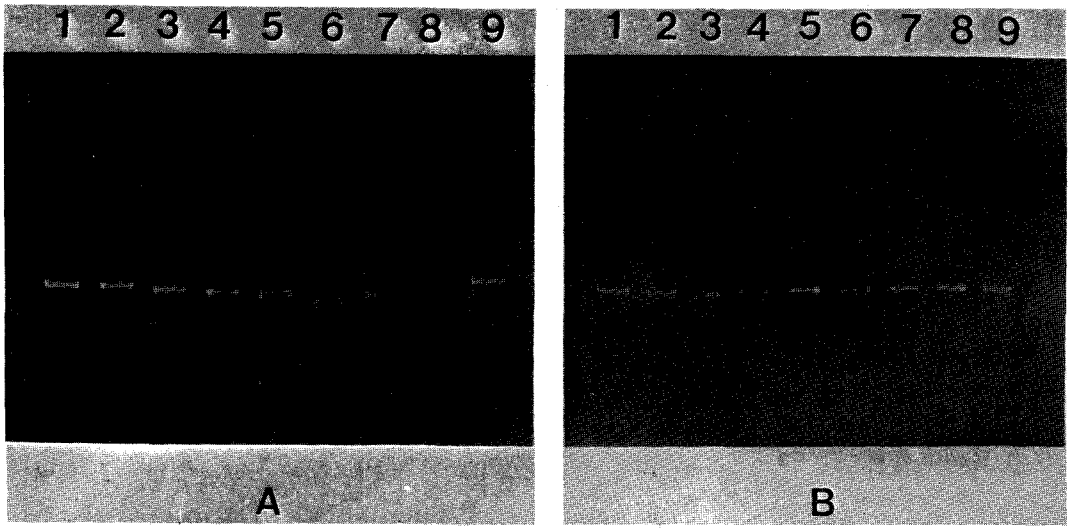

Fig. 3. Electrophoresis of CDDP-treated form Ⅲ pBR322 DNA in 1% agarose gel.

Electrophoresis was performed in horizontal submarine gel at 5V/cm for 6hr. The DNA bands were stained with 0.5μg/ml of EtBr for 30min. Lane 1 and 9 ; control, lane 2 ; 10μM CDDP(TDDP), lane 3 ; 25μM CDDP(TDDP), lane 4 ; 50μM CDDP(TDDP), lane 5 ; 100μM CDDP(TDDP), lane 6 ; 200μM CDDP(TDDP), lane 7 ; 500μM CDDP(TDDP), lane 8 ; 1mM CDDP(TDDP). Panel A ; CDDP, panel B ; TDDP.

within a cruciform junction DNA(Gough and Lilley, 1985). The migration of cruciform DNA was inversely proportional to a function of gel concentration. The CDDP-treated form Ⅲ DNA was also run in 2% agarose gel. CDDP-treated DNA migrated slowly compared to control DNA(Fig. 4, lane 2) for the pore size of 2% gel is relatively small compared to that of 1% agarose gel. This result confirms that the fast migrating pattern of CDDP-treated form Ⅲ DNA in 1% agarose gel was due to the conformational change.

S1 nuclease digestion

To investigate if CDDP induces denaturation on double strand DNA, single strand DNA specific S1 nuclease was employed. Since high concentration of S1 nuclease is also able to digest double strand DNA as well, the least concentration of S1 nuclease that would not digest double strand DNA, was determined. Form Ⅲ pBR322 DNAs digested with different concentrations of S1 nuclease were separated in 1% agarose gel. Lane 3 of Figure 5 showes that at 0.5unit of S1 nuclease the DNA band intensity was not changed compared to control while at the S1 nuclease concentration greater than 2.5 unit, the DNA band intensity were greatly reduced(Fig.

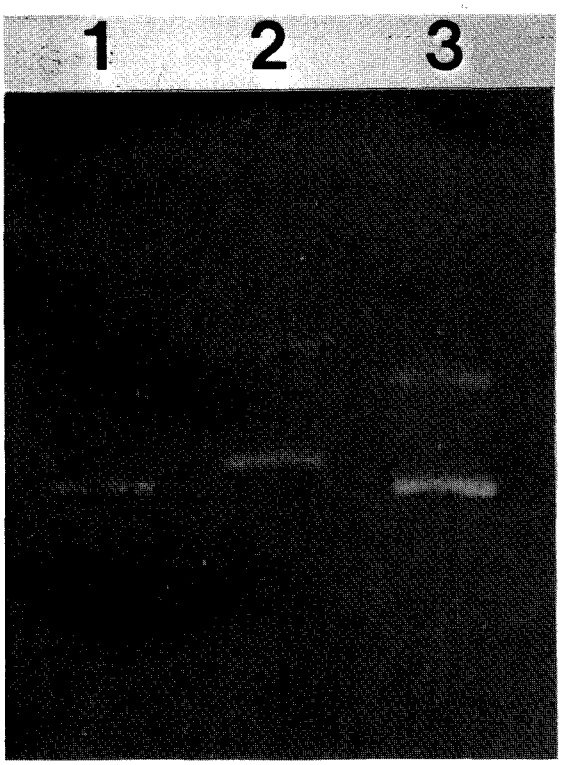

Fig. 4. Electrophoresis of CDDP-treated form Ⅲ pBR322 DNA in 2% agarose gel.

Electrophoresis was performed in horizontal submarine gel at 5V/cm for 3hr. The DNA bands were stained with 0.5μg/ml of EtBr for 30min. Lane 1 and 3 ; control, lane 2 ; 100μM CDDP.

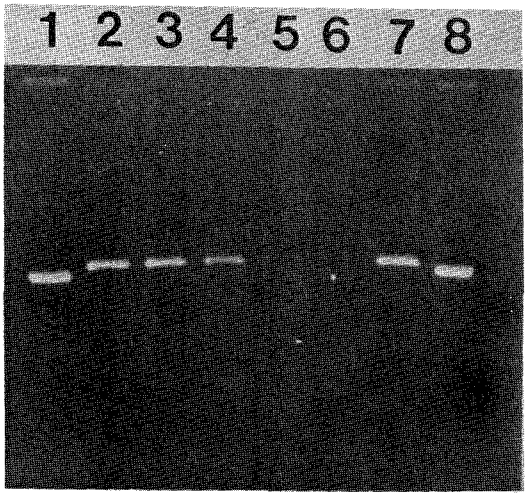

Fig. 5. Electrophoresis of S1 nuclease-treated form Ⅲ pBR322 DNA.

Form Ⅲ pBR322 DNAs were treated with increasing concentrations of S1 nuclease at 37℃ for 30min. The reaction was stopped by adding 0.5M EDTA and the DNAs were separated in 1% agarose gel at 5V/cm for 3hr. The DNA bands were stained with 0.5㎍/ml of EtBr for 30min. Lane 1 and 8 ; control, lane 2 and 7 ; control(S1 buffer), lane 3 ; 0.5unit S1, lane 4 ; 2.5unit S1, lane 5 ; 25unit S1, lane 6 ; 250unit S1.

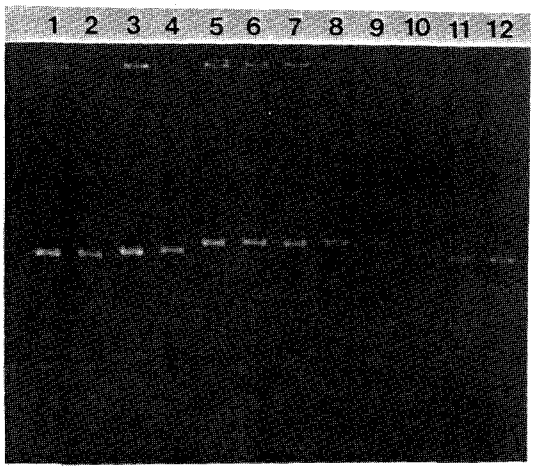

Fig. 6. Electrophoresis of CDDP-treated form Ⅲ pBR322 DNA digested with S1 nuclease.

CDDP-treated DNA was digested with 0.5unit of S1 nuclease at 37℃ for 30min. The reaction was stopped by adding 0.5M EDTA and the DNAs were separated in 1% agarose gel at 5V/cm for 4hr. The DNA bands were stained with 0.5㎍/ml of EtBr for 30min. Lane 1 and 12 ; control, lane 2 and 11 ; control(0.5unit S1), lane 3 ; 5㎛M CDDP, lane 4 ; 5㎛M CDDP(0.5unit S1), lane 5 ; 50㎛M CDDP, lane 6 ; 50㎛M CDDP(0.5unit S1), lane 7 ; 100㎛M CDDP, lane 8 ; 100㎛M CDDP(0.5unit S1), lane 9 ; 500㎛M CDDP, lane 10 ; 500㎛M CDDP (0.5unit S1)

5, lane 4, 5, and 6). These results indicate that 0.5unit of S1 nuclease would not digest double strand DNA significantly.

The form Ⅲ pBR322 DNAs treated with different concentrations of CDDP were digested with 0.5unit of S1 nuclease. Figure 6 showed that the CDDP-treated DNA band intensity was reduced after digestion with S1 nuclease. The band intensity was greatly reduced after treat the DNA with 50㎛M of CDDP(lane 5 and 6), 100 ㎛M of CDDP(lane7 and8), and 500㎛M of CDDP (lane 9 and 10). These results suggest that CDDP adduction to double strand DNA induces locally denatured region that would be susceptible to single strand DNA specific nuclease S1.

Acknowledgments

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cis-Diamminedichloroplatinum(Ⅱ)에 의한 pBR322 DNA의 변성과 구조 변화

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초록 : CDDP를 처리한 pBR322 DNA로 형질변환된 대장균 LE392를 ampicillin이 포함된 한천평판배지위에 도말시켰다. Ampicillin을 함유하고 있는 평판배지위에 형성된 집락수는 13.3 μM의 CDDP를 처리한 뒤에는 검출되지 않을 정도로 감소하였다. CDDP를 처리한 pBR322 DNA는 외가닥 DNA에 특이성이 있는 S1 핵산분해효소에 의해 절단되었고 아가로즈 겔 전기영동상에서 이동 유형이 변했다. 이러한 결과에 의하면 CDDP가 pBR322 DNA에 반응하여 이중나선의 변성과 궁극적으로는 ampicillin 저항성 유전자를 불활성화시키는 구조 변화를 일으키는 것 같다.