SOME EVIDENCE FOR INTERACTION OF D-CYCLOSERINE WITH DNA

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D-Cycloserine showed a decreased lethality against paraquat-treated *E. coli* than against non-treated cells. *E. coli* K12 mutants which were deficient in DNA repair were more sensitive than the wild type strain to D-cycloserine. Furthermore, D-cycloserine caused PM2 DNA cleavage in the presence of Fe^{2+}, and the cleavage was strongly inhibited by singlet oxygen scavengers. These results indicate that D-cycloserine interacts with DNA and an active oxygen species is involved in the interaction.

In the previous paper[1], we described a simple screening method for antitumor antibiotic producing oxygen radicals. Using this method, during the course of our screening for new antitumor antibiotics an antibiotic, MMX-1, was found. In the assay plate of this screening method, the diameter of the inhibition zone of MMX-1 was decreased by the supplementation of paraquat, and MMX-1 was more toxic to *rec*$^-$ strain than to *rec*$^+$ strain. To our surprise, this compound was identified as D-cycloserine (D-4-amino-3-isoxazolidone)[2]. Therefore, we investigated the reason why D-cycloserine was found using the screening system, because this antibiotic has been well known to inhibit the synthesis of bacterial peptidoglycan.

In this paper, we report on evidence for the interaction of D-cycloserine with DNA.

Materials and Methods

Assay of Resistance towards D-Cycloserine

Resistance to D-cycloserine was tested as described previously[3]. In this case, 0.2 mg/ml of D-cycloserine was added instead of bleomycin A_2. *Escherichia coli* K12 was grown in Trypticase soy-yeast extract (TSY) medium containing 2% Trypticase soy broth (BBL) and 0.5% yeast extract (Oriental) in a L-shaped tube at 37°C on a shaker at 200 rpm. Cell-free extracts were prepared and assayed for superoxide dismutase, catalase and peroxidase, as previously described[3]. Protein was estimated by the Bio-Rad protein assay method[4].

Antibacterial Activity of D-Cycloserine against *E. coli* K12 Mutants

The following strains including the relevant genotypes were used: *E. coli* K12 KL-16 *rec*$^+$ *thi-1* and its isogenic derivative LC248 *recA1 thi-1*; *E. coli* K12 W3110 and its almost isogenic P3478 *polA1 thy* and AB2494 *lex-1 thr-1 metB1 his4 proA2*. All strains were supplied by Dr. Y. HIROTA, National Institute of Genetics, Mishima, Japan. TSY medium was supplemented with 20 μg/ml of thymine, threonine, methionine, histidine and proline and 1 μg/ml of thiamine hydrochloride according to the requirements of mutants. Ten milliliters of basal layer containing 1.5% agar were solidified in a Petri dish (9 cm in diameter), and 4.5 ml of the melted top layer containing test strains (one drop of suspensions of an overnight-shake culture at 37°C to 50 ml of the medium containing 1.5% agar) were overlaid. A cup diffusion method was used for the quantitative assay of the bacterial growth. After incubation at 30°C for 20 hours, the diameter of the inhibitory zone was measured. D-Cycloserine was dissolved in deionized water.

DNA Cleavage and Gel Electrophoresis

The reaction mixture contained 0.15 μg PM2 DNA (generous gift of Prof. M. HORI, Showa College of Pharmaceutical Sciences) and 0.05 mM $FeCl_2$ in 50 μl of 90 mM tris-borate buffer (pH 8.3). It was incubated at 37°C for 3 hours in the presence or absence of D-cycloserine and/or metal ions as indicated. Ten microliters of 90 mM tris-borate buffer (pH 8.3) containing 3Na-EDTA (4 mM), glycerol (50%, v/v) and bromophenol blue (0.1%) were added to the reaction mixture. Electrophoretic analysis was performed as described by HOTTA *et al.*[5] The agarose (0.7%, Seakem ME) gels were run in the presence of 0.5 μg/ml ethidium bromide. The stained bands were visualized using a UV lamp and photographed under UV light through UV and orange filters using Kodak Tri-X film. Water was deionized and highly purified by a Milli R/Q water purifier (Millipore Corporation).

Measurement of Singlet Oxygen-scavenging Activity

Singlet oxygen-scavenging activity was measured according to the method of KELLOG and FRIDOVICH[6] based on testing the peroxidation of linolenate by singlet oxygen produced photochemically.

The complete mixture containing 0.01 mM rose bengal, 3 mM linolenic acid and 10% dimethoxy ethane in 50 mM potassium phosphate buffer (pH 8.1) in a total volume of 3 ml was irradiated with a 15 watt fluorescent lamp. The tube was surrounded by aluminum foil which reflected the light. Either dialyzed or non-dialyzed sample of cell-free extracts corresponding to 1.5 mg of protein was added to the complete reaction mixture. The reaction was terminated by acidifying the mixture to pH 4.0 with 1 N HCl, and the mixture was extracted with 0.6 ml of chloroform and the extracts concentrated under the reduced pressure. Twenty microliters of the extracts was applied, as spots, to a silica gel $60F_{254}$ plate (Merck) and developed in light petroleum - benzene - acetone - acetic acid (10: 10: 10: 1, v/v). Hydroperoxide spots were detected by spraying 0.1% *N,N*-dimethyl-*p*-phenylenediamine in chloroform - acetic acid - water (5: 5: 1, v/v)[7]. All spots were visualized by spraying with ethanol - sulfuric acid (1: 1, v/v), and then heating the plate at 130°C for 5 minutes.

Chemicals

Crystalline D-cycloserine, paraquat (methyl viologen, 1,1′-dimethyl-4,4′-bipyridinium dichloride), superoxide dismutase (EC 1.15.1.1, bovine blood type I 3,000 U/mg protein) and catalase (EC 1.11.1.6, bovine liver, 11,000 U/mg protein) were purchased from Sigma Chemical Co., linolenic acid (approximately 99% pure by TLC) was purchased from PL-Biochemicals Inc. All other chemicals were of reagent grade.

Results

Correlation between the Level of Superoxide Dismutase and Resistance to D-Cycloserine

Escherichia coli whose content of superoxide dismutase was augmented by paraquat treatment exhibited increased resistance to antibiotics which generated oxygen radical species.[8] In the disc-diffusion method previously described[1], the diameter of the inhibitory zone of D-cycloserine was decreased by the supplementation of paraquat into the assay plate. This observation suggested that D-cycloserine would generate active oxygen radical species.

To confirm this property, the correlation between the induction of superoxide dismutase in *E. coli* by paraquat treatment and the tolerance of this organism to D-cycloserine was investigated. *E. coli* K12 was grown for 2 hours in TSY medium containing 0.0, 0.1 and 1.0 mM paraquat in order to modify the cellular content of superoxide dismutase, and suspended in glucose minimal medium containing 0.5 mg/ml of chloramphenicol and 0.2 mg/ml of D-cycloserine. At indicated intervals samples were taken for the assay of surviving cells. In addition, the specific activities of superoxide dismutase, catalase and peroxidase of the cell-free extracts of *E. coli* K12, which were grown for 2 hours in TSY media containing 0.0, 0.1 and 1.0 mM paraquat, were estimated.

Table 1. Effect of paraquat on level of defensing activities against oxygen toxicity.

U/mg protein	Paraquat (methyl viologen, mM)		
	0	0.1	1.0
Superoxide dismutase	26.0	100	155
Catalase	18.3	25.2	28.7
Peroxidase	0.3	0.4	0.35

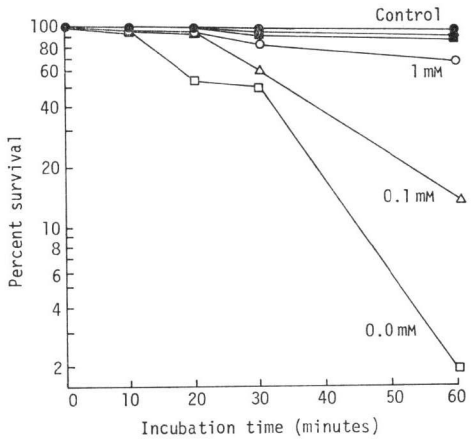
Enzyme activities were measured on the cell-free extracts of *E. coli* K12, which were grown for 2 hours in TSY media containing 0.0, 0.1 and 1.0 mM paraquat, according to the methods described in the text.

Fig. 1. Correlation between paraquat concentration and resistance to D-cycloserine.

E. coli K12, which were grown for 2 hours in TSY media containing 0.0, 0.1 and 1.0 mM paraquat, were collected, washed, and suspended to 10^7 cells/ml in glucose minimal medium containing 0.5 mg/ml of chloramphenicol and 0.2 mg/ml of D-cycloserine and were then incubated at 37°C. At indicated intervals the cells were taken, diluted, and plated onto TSY agar medium, and viable counts were carried out after overnight incubation at 37°C.

The control were performed in the same way, but excepting D-cycloserine.

□, Cells grown in the absence of paraquat (■, control). △, Cells grown in the presence of 0.1 mM paraquat (▲, control). ○, Cells grown in the presence of 1.0 mM paraquat (●, control).



The results, as shown in Table 1 and Fig. 1, clearly demonstrated that a prior exposure of *E. coli* K12 to paraquat, with its concomitant induction of superoxide dismutase, increased resistance to D-cycloserine in proportion to the concentration of paraquat. This observation indicates that superoxide anion (O_2^-) and/or other active oxygen species derived from O_2^- is more or less participating in the bactericidal activity by D-cycloserine.

Response to D-Cycloserine of *E. coli* Mutants Deficient in DNA Repair

It is well-known that a *rec*$^-$ mutant is more susceptible than a *rec*$^+$ strain to agents which interact with DNA. In a previous paper, D-cycloserine showed a stronger inhibition against *rec*$^-$ than against *rec*$^+$ strains. We studied, therefore, the effect of D-cycloserine against several kinds of *E. coli* K12 mutants deficient in DNA repair.

Fig. 2. Response to D-cycloserine of microorganisms deficient in DNA repair (cup-diffusion assay).

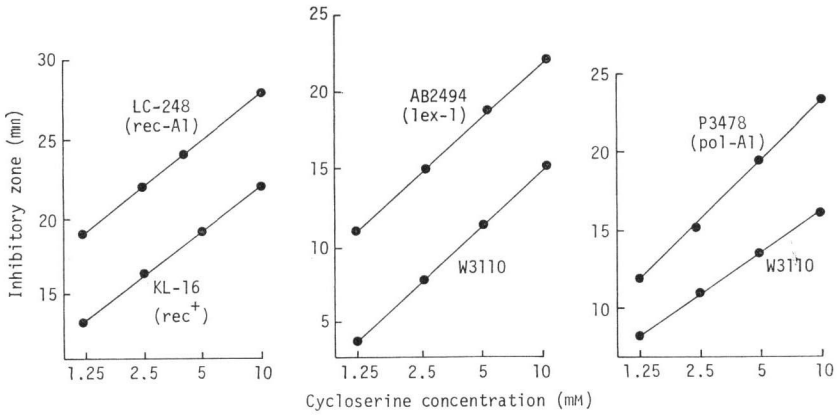


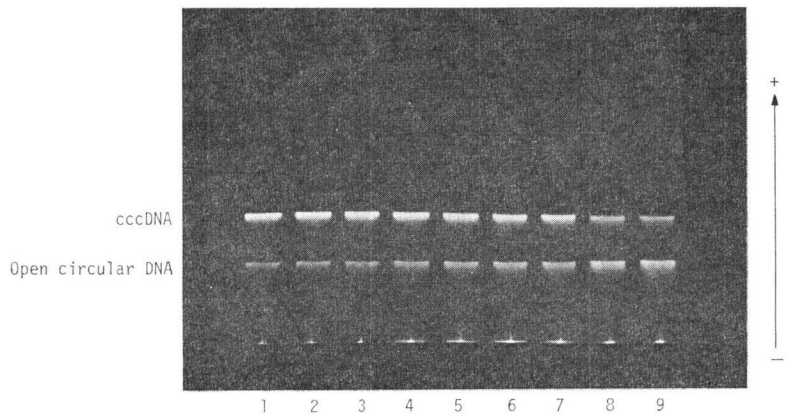
Fig. 2 shows the dose-response curves of wild type strains and mutants defective in DNA repair against D-cycloserine. *E. coli* K12 with *recA1*, *lex-1* and *polA1* mutations was more sensitive than wild type to D-cycloserine. It was suggested, therefore, that D-cycloserine could interact with the DNA molecule *in vivo*.

Interaction of D-Cycloserine with PM2 DNA

In the preceding sections, D-cycloserine was suggested not only to generate active oxygen species but also to act on the DNA molecule in *E. coli* K12. Therefore, we studied the interaction of D-cycloserine with covalently closed circular PM2 DNA. As illustrated in Fig. 3, lane 1, PM2 phage DNA showed two major bands on agarose gel electrophoresis. According to YAGI *et al.*,[9] the fast moving band corresponded to the native form of covalently closed circular DNA (cccDNA), and the slow moving band the open circular form. As shown in Fig. 3, lane 2 and 3, D-cycloserine (5 mM) or Fe^{2+} (0.05 mM) alone was not injurious to PM2 DNA. As shown in Fig. 3, lane 4~9, D-cycloserine with Fe^{2+} decreased the cccDNA and increased the open circular form. This conversion was proportional to the concentration of D-cycloserine. The result indicates that D-cycloserine reacts with DNA in the presence of Fe^{2+} and causes single strand scission of PM2 cccDNA.

The reducing agents, sodium borohydride, ascorbic acid, sodium hydrosulfite, 2-mercaptoethanol and dithiothreitol, in 0.1 mM concentration did not influence the reaction of D-cycloserine with DNA, and other metal ions such as Fe^{3+}, Co^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+} in 0.1 mM could not replace Fe^{2+}.

Fig. 3. Induction of single strand scission in PM2 DNA by D-cycloserine in the presence of Fe^{2+}.
1) Drug free control. 2) 5 mM D-cycloserine. 3) 0.05 mM Fe^{2+}. 4) 3) +0.1 mM D-cycloserine. 5) 3) +0.5 mM D-cycloserine. 6) 3) +1 mM D-cycloserine. 7) 3) +2 mM D-cycloserine. 8) 3) +5 mM D-cycloserine. 9) 3) +10 mM D-cycloserine.

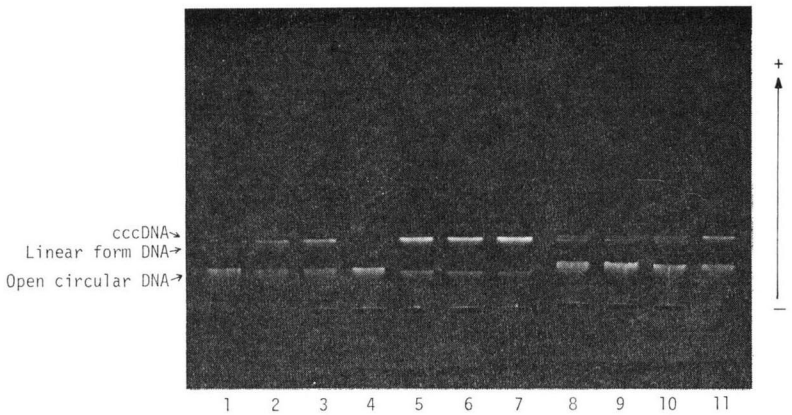


Effect of Enzymes and Radical Scavengers on DNA Cleavage by D-Cycloserine in the Presence of Fe^{2+}

The influences of enzymes and several radical scavengers on the *in vitro* cleavage of DNA by D-cycloserine in the presence of Fe^{2+} were tested. The effect of a high concentration of D-cycloserine (20 mM) and a long incubation time (4 hours) was studied to obtain significant results. Under these conditions, as shown in Fig. 4, the intermediate band corresponding to the linear form of DNA was produced between cccDNA and open circular form[9].

Fig. 4. Effects of Fe^{2+}-chelators and radical scavengers on the induction of single strand scission in PM2 DNA.

1) Complete mixture (20 mM D-cycloserine, 0.05 mM Fe^{2+}). 2) +1 mM α,α'-Dipyridil. 3) +1 mM *o*-Phenanthroline. 4) +10 mM DABCO. 5) +1 mM β-Carotene. 6) +1 mM 2,5-Diphenylfuran. 7) +1 mM DL-α-Tocopherol. 8) +10% (v/v) 1,2-Dimethoxyethane. 9) +0.1 M *tert*-Butyl alcohol. 10) +10% (v/v) Methyl alcohol. 11) +1 mM 4-Nitrocathecol.



Catalase (10 μg/ml) slightly inhibited DNA strand scission: the heat-inactivated enzyme had no effect. Superoxide dismutase (50 μg) also did not inhibit DNA cleavage (data not shown).

α,α'-Dipyridil (1 mM) and *o*-phenanthroline (1 mM) (chelators of Fe^{2+}) inhibited DNA strand scission. 4-Nitrocatechol (1 mM) (a general inhibitor for non-heme iron dioxygenases)[10] also inhibited DNA strand scission. DNA cleavage caused by D-cycloserine in the presence of Fe^{2+} was completely reversed by singlet oxygen scavengers such as β-carotene (1 mM), 2,5-diphenylfuran (1 mM) and DL-α-tocopherol (1 mM), but not by 1,4-diazabicyclo[2,2,2]octane (DABCO, 10 mM). 1,2-Dimethoxyethane (10%, v/v), *tert*-butyl alcohol (0.1 M) and methyl alcohol (10%, v/v) (scavengers of hydroxyl radical) did not inhibit DNA strand scission.

These results suggest that singlet oxygen may be involved in PM2 DNA strand scission by D-cycloserine.

Singlet Oxygen-scavenging Activity of Cell Extracts of *E. coli* Exposed to Paraquat

As shown in the preceding section, D-cycloserine cleaved DNA in the presence of ferrous ion aerobically and this cleavage was strongly inhibited by several scavengers of singlet oxygen. It seemed, therefore, that a singlet oxygen probably was involved in the bactericidal activity by D-cycloserine. We measured, therefore, the levels of scavenging activity of singlet oxygen of cell-free extracts of paraquat-treated *E. coli* to explain more precisely the increased tolerance of paraquat-treated *E. coli* against D-cycloserine, as shown in Fig. 1.

Both dialyzed and non-dialyzed samples of cell-free extracts of *E. coli* K12, which were grown for 2 hours in TSY media containing 0.0, 0.1 and 1.0 mM paraquat, were prepared and measured for singlet oxygen-scavenging activity according to the method based on testing the inhibition of peroxidation of linolenate by singlet oxygen produced photochemically.

As shown in Fig. 5, the production of hydroperoxide, which was formed by irradiation of a solution containing rose bengal, was depressed by the addition of cell-free extracts of paraquat-treated *E.*

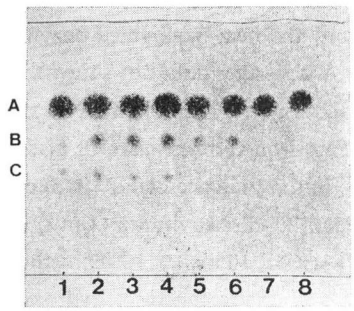
Fig. 5. Linolenate peroxidation by a photochemical source of singlet oxygen.

The complete mixture containing 0.01 mM rose bengal, 3 mM linolenic acid and 10% 1,2-dimethoxyethane in 50 mM potassium phosphate buffer (pH 8.1) in a total volume of 3 ml was irradiated by exposure to 15 watt fluorescent lamp in a reflective enclosure. Either dialyzed or non-dialyzed sample of cell-free extracts corresponding to 1.5 mg of protein was added to the complete mixture.

The conditions were modified as follows:

1) No irradiation of complete mixture. 2) Complete system. 3) Plus non-dialyzed cell-free extracts of *E. coli* grown in the absence of paraquat. 4) Dialyzed sample of lane 3. 5) Plus non-dialyzed cell-free extracts of *E. coli* grown in the presence of 0.1 mM paraquat. 6) Dialyzed sample of lane 5. 7) Plus non-dialyzed cell-free extracts of *E. coli* grown in the presence of 1.0 mM paraquat. 8) Dialyzed sample of lane 7.

(Spot A) residual linolenic acid; (spot B) hydroperoxide; (spot C) unknown.



coli K12, and the degree of the depression was related to the concentration of paraquat to which the *E. coli* was exposed. Dialysis of cell-free extracts did not alter the degree of depression. In conclusion, a prior exposure of *E. coli* to paraquat caused a marked increase of singlet oxygen-scavenging activity, and this activity was suggested to be due to high molecular weight substances.

Discussion

In this paper, we described the following; (1) Paraquat-treated cells of *E. coli* K12 which had higher levels of superoxide dismutase were more resistant to D-cycloserine than non-treated cells which had low cellular levels of this enzyme. (2) A prior exposure of *E. coli* to paraquat caused an enhancement of cellular levels of singlet oxygen-scavenging activity. (3) D-Cycloserine was more toxic against *E. coli* mutants deficient in DNA repair than against the wild type strain. (4) D-Cycloserine caused single strand scission of covalently closed circular PM2 DNA in the presence of ferrous ion, and DNA cleavage was strongly inhibited by scavengers of singlet oxygen.

In summary, our results indicate that D-cycloserine cleaved DNA molecules and active oxygen species are involved in this interaction, although the generation mechanism is not known.

It is difficult, however, to judge from the results obtained in the present study to what extent this interaction of D-cycloserine with DNA is contributing to the antimicrobial activity of D-cycloserine.

It is noteworthy that the DNA strand scission by D-cycloserine is not inhibited by superoxide dismutase. A similar result has been obtained in the case of DNA breakage caused by bleomycin[11,12].

Neocarzinostatin, an antitumor protein, also generates active oxygen-radicals which causes DNA strand scission.[13] In this case, the chromophore of the antibiotic is involved in this reaction. Furthermore its five membered cyclic carbonate structure is similar to that of cycloserine[14]. This is the first finding that a simple amino acid like D-cycloserine interacts with the DNA molecule resulting in DNA strand scission. We examined the antitumor activity of D-cycloserine against mouse leukemia L-1210, but no effect was observed even at 400 mg/kg/day (i.p.-i.p., data not shown).

Acknowledgements

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