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Study of the Mechanism of Action of *p*-Chloromercuribenzoate on Endonuclease from the Bacterium *Serratia marcescens*

M. N. Filimonova[1]*, V. P. Gubskaya[2], I. A. Nuretdinov[2], M. J. Benedik[3], N. A. Cherepanova[1], and I. B. Leshchinskaya[1]

[1]Department of Microbiology, Kazan State University, ul. Kremliovskaya 18, Kazan, 420008 Russia; E-mail: maria.filimonova@ksu.ru
[2]Arbuzov Institute of Organic and Physical Chemistry, Kazan Scientific Center, Russian Academy of Sciences, ul. Arbuzova 8, Kazan, 420088 Russia; fax: (2-843) 752-253; E-mail: in@iopc.ksn.ru
[3]Department of Biochemical and Biophysical Sciences, University of Houston, Houston, TX 77204-5934, USA; fax: (713) 743-8351; E-mail: benedik@uh.edu

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Abstract—The mechanism of action of *p*-chloromercuribenzoate (PCMB) on *Serratia marcescens* nuclease was investigated. The analysis showed that PCMB forms complexes with DNA. Binding of $C_7H_5O_2Hg^+$ to DNA changes the secondary structure of the DNA. These changes alter the enzymatic activity of *S. marcescens* nuclease, which was previously found to be sensitive to the secondary structure of the substrates. The nuclease activity was either suppressed or stimulated in the presence of PCMB depending on the $C_7H_5O_2Hg^+$ to nucleotide equivalent ratio. Binding of $C_7H_5O_2Hg^+$ to DNA did not form an abortive enzyme–substrate complex. Binding of Mg^{2+} to the $C_7H_5O_2Hg$–DNA complex caused appropriate changes in secondary structure of the substrate. Since Mg^{2+} and $C_7H_5O_2Hg^+$, though differing in the type of metal cation, are similar in their mechanisms of influence on enzymatic activity of *S. marcescens* nuclease, the identity of other metal-containing effectors in their mechanism of action on *Serratia marcescens* nuclease is assumed.

Key words: endonuclease, *Serratia marcescens*, CD, PCMB, DNA

Endonuclease (EC 3.1.30.2) from the bacterium *Serratia marcescens* is an enzyme of known structure [1, 2] and mechanism of action [3]. Its physicochemical and biochemical properties have been examined in detail [4-7]. Various molecular forms of the nuclease have been isolated and characterized [8-12].

Previously we found that PCMB suppresses the activity of the endonuclease and concluded that the protein contains SH-groups [6]. Further examination showed that the endonuclease contains no SH-group [8]. Thus, the effect of PCMB on the endonuclease activity remained unclear.

Detailed study of the effect of Mg^{2+} on the enzyme showed that the endonuclease activity strictly depends on the formation of a Mg^{2+}–DNA complex [11]. Because Hg^{2+} is also known to interact with polynucleotides [13-16], a similarity in mechanisms of action of Mg^{2+} and PCMB on the endonuclease was considered.

Although data on DNA binding with mercury cations are consistent [13-16], most studies have been carried out using mainly $HgCl_2$ or $Hg(ClO_4)_2$ as the mercury containing compounds. PCMB, representing another type of chemical compounds, is better known as a specific reagent reacting with SH-groups in proteins [17].

The lack of knowledge concerning the interaction of PCMB with DNA and our data showing that PCMB affects the activity of the *S. marcescens* endonuclease prompted us to investigate the ability of PCMB to interact with polynucleotides and to determine the correlation between the expected interaction and changes in the endonuclease activity.

We studied the effect of PCMB over a wide range of concentrations on both the endonuclease activity and DNA conformation. Since the endonuclease is represented by several isoforms, isoforms Sm1 and Sm2 differing in their physicochemical and biochemical properties and found in most strains of *S. marcescens* were used for the studies [8-11].

* To whom correspondence should be addressed.

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MATERIALS AND METHODS

Production of the nuclease isoforms. The nuclease was isolated from the fermentation broth of *S. marcescens* B10M1 [18] and the isoforms were separated by previously elaborated methods [6, 19] using anion- and cation-exchange chromatography on DEAE-cellulose DE-32 (Reanal, Hungary), phosphocellulose P-11, and DEAE-cellulose DE-52 (Whatman, UK). The homogeneity of the enzyme preparations has been previously described [8].

Nuclease activity assay. Since both amino groups of Tris are known to interact with Hg^{2+} and Cl^- ions easily form complexes with Hg^{2+}, negating the formation of Hg^{2+} complexes with polynucleotides [14], the effects of PCMB on the endonuclease activity were studied in borate buffer instead of the traditionally used Tris-HCl buffer. One milliliter of the assay mixture contained 1 mg of DNA from chicken erythrocytes (Reanal, Hungary), isoform Sm1 (0.21 nM) or Sm2 (0.26 nM), 0.1 M borate buffer (pH 8.5), and 10^{-2}-10^{-10} M PCMB. When Mg^{2+} was needed, the assay mixture additionally contained 30 mM $MgSO_4$. The reactions mixtures were incubated at 37°C and stopped by the addition of cooled 4% $HClO_4$ when less than 75% of the substrate had been converted to acid soluble fragments. The precipitate was removed by centrifugation, and the absorbance of the supernatant was measured at 260 nm. One unit of nuclease activity was defined as the amount of enzyme causing an increase of 1.0 A_{260}/h at 37°C. The concentration of the isoforms were calculated based on their molecular masses [9] and molar extinction coefficient of 47,292 $M^{-1}\cdot cm^{-1}$ [7]. The nuclease activity in the absence of PCMB was taken as 100%.

Reversibility of the effect of PCMB on the endonuclease. This was studied by determining the rate of hydrolysis of herring testes DNA (type XIV, Sigma, USA) by the hyperchromicity effect using a Milton Roy Spectronic 3000 diode-array spectrophotometer with the Rate Analysis software package (SLM/Amico, USA). Isoform Sm2 (8 μM) was added to assay mixture containing 10 mM potassium phosphate buffer (pH 7.04), 125 μg/ml DNA (0.3625 mM nucleotide equivalents), and 0 or 5 mM PCMB. After measuring the rate of hydrolysis (for approximately 5 min), 1000-fold diluted samples were added to a further 10-fold volume of newly prepared assay mixture containing 80 mM Tris-HCl buffer (pH 8.5), 125 μg/ml DNA, and 2.5 mM Mg^{2+}. The activity was monitored.

UV spectra of DNA were measured in the 210-350 nm range using a Specord M-40 spectrophotometer (Germany). The samples contained 0.002% chicken erythrocyte DNA, 2 mM borate buffer (pH 8.5), 0 or $2\cdot10^{-4}$-$2\cdot10^{-9}$ M PCMB, and 0.6 mM Mg^{2+}. UV spectra of similar samples in the absence of DNA were used as the baselines.

Circular dichroism spectra of DNA were measured at room temperature using a Jasco-J 500 A spectrometer (Japan Spectroscopic, Japan). The samples contained 0.05% of chicken erythrocyte DNA, 50 mM borate buffer (pH 8.5), and 0 or $5\cdot10^{-3}$-$5\cdot10^{-10}$ M PCMB. After addition of PCMB, the DNA preparations were preincubated for 15 min at room temperature before measuring the CD spectra.

The ratios of components in the samples prepared for UV or CD spectra measurements were identical to their ratios in the assay mixture used for examination of the effect of PCMB on the endonuclease activity.

RESULTS

Data on the activity of the endonuclease in the presence of PCMB and activating Mg^{2+} ions are shown in Fig. 1a. As seen from the figure, PCMB at concentrations of 0.1-10 mM or 1-10 μM caused a decrease (by 25-85%) or, in the opposite, increase (by 5-45%) of the nuclease activity compared with the samples in the absence of PCMB. Activation of isoform Sm1 (line *1*) was 20-30% higher than the activation of isoform Sm2 (line *2*).

Removing Mg^{2+} from the assay mixture cancelled the difference between the effects of PCMB on the two isoforms (Fig. 1b) but did not alter the common trends in the dependence of the endonuclease activity on PCMB concentration (Fig. 1, a and b).

The suppression of the endonuclease activity caused by PCMB was reversible. If an aliquot of the used assay mixture containing the endonuclease was transferred to a newly prepared assay mixture, the endonuclease activity was almost completely recovered (table).

Because the interaction of Hg^{2+} with DNA is known to cause pronounced changes in the physical properties of the DNA, including the UV and CD spectra [13-14, 20, 21], we used absorbance and circular dichroism spectroscopy to analyze the interaction of PCMB with DNA.

UV spectra of DNA preparations containing different amounts of PCMB suggest that PCMB interacts with these polynucleotides (Fig. 2). The addition of PCMB caused a small but reproducible violet shift of the λ_{max} and λ_{min} (by 2-3 nm) in the spectra and changed the magnitude of the mentioned absorbances.

The differences in CD spectra of DNA preparations differing in PCMB concentration also indicated an interaction of PCMB with the polynucleotide independently of the presence of Mg^{2+} (Fig. 3).

As seen from Fig. 3a, the CD spectrum of DNA in the absence of PCMB is the spectrum of B-form DNA [22, 23]; it exhibits large and small positive bands centered at 275 and 217 nm, respectively, a large negative band centered at 242 nm, and intersections of the abscissa at 226 and 256 nm. The lack of a band at 290-300 nm confirms the absence of protein contamination [24] in the analyzed DNA preparation.

Addition of PCMB to the DNA preparations caused a red shift of the large positive band and changed the magnitude of its specific ellipticity in CD spectra *2*, *3*, and *5*-

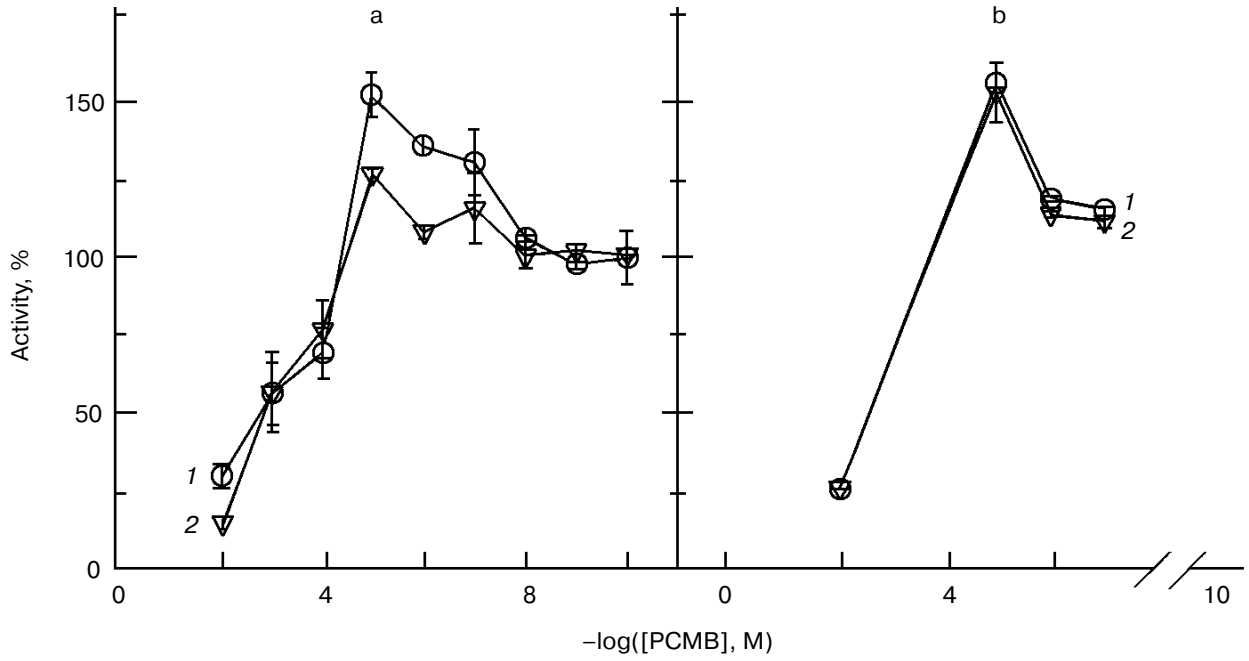

Fig. 1. Dependence of the DNase activities of isoforms Sm1 (*1*) and Sm2 (*2*) on PCMB concentration measured in the presence (a) or in the absence (b) of Mg^{2+}.

8 (Fig. 3, a and b). The spectrum produced at the highest concentration of PCMB (line *2*) was separated from the other spectra by the drastic changes of the above-mentioned characteristics. Compared with the control (line *1*), the red shift of the spectrum (line *2*) was more than 7 nm and the magnitude of the specific ellipticity was twofold decreased. At the other concentrations of PCMB (lines *3*, *5*-*8*), the red shift of λ_{max} was about 3 nm and the specific ellipticity varied in the range of 10%.

Additions of Mg^{2+} to the samples did not influence the common trends in the spectra and also caused variations in the CD spectra (Fig. 3b). In the presence of Mg^{2+} the magnitude of the specific ellipticity in the large positive band decreased by about 18% and the PCMB-induced red shift increased.

DISCUSSION

The changes in the endonuclease activity under the influence of PCMB (Fig. 1) confirmed the well-known decrease in the enzymatic activity of *S. marcescens* nuclease in the presence of 10^{-2}-10^{-3} M PCMB and revealed a previously unknown activation of the endonuclease by PCMB.

The presence of Mg^{2+} did not change in principal the effect of PCMB on the endonuclease activity. This is shown by the similarity of the curves displaying the dependence of the activities of isoforms Sm1 and Sm2 on PCMB concentration in the presence and in the absence of Mg^{2+} (Fig. 1, a and b, lines *1* and *2*).

During the examination of the reversibility of the effect of PCMB on the endonuclease activity, decreasing

Reversibility of the effect of PCMB on the activity of *S. marcescens* endonuclease

$C_7H_5O_2Hg^+$/nucleotide equivalent ratio	Activity of endonuclease			
	in the absence of PCMB		in the presence of PCMB	
	%	OD/min × 10^2	%	OD/min × 10^2
13.8	100 ± 3.7	2.7 ± 0.1	22.2 ± 3.7	0.6 ± 0.1
0.00138*	100 ± 1.4	21.1 ± 0.3	83.9 ± 7.1	17.7 ± 1.5

* After measuring the activity at $C_7H_5O_2Hg^+$/nucleotide equivalent ratio of 13.8, aliquots were taken from the used assay mixture, 1000-fold diluted with distilled water, and added to a further 10-fold volume of newly prepared assay mixture containing 125 μg/ml of the DNA, 80 mM Tris-HCl buffer (pH 8.5), and 2.5 mM Mg^{2+}.

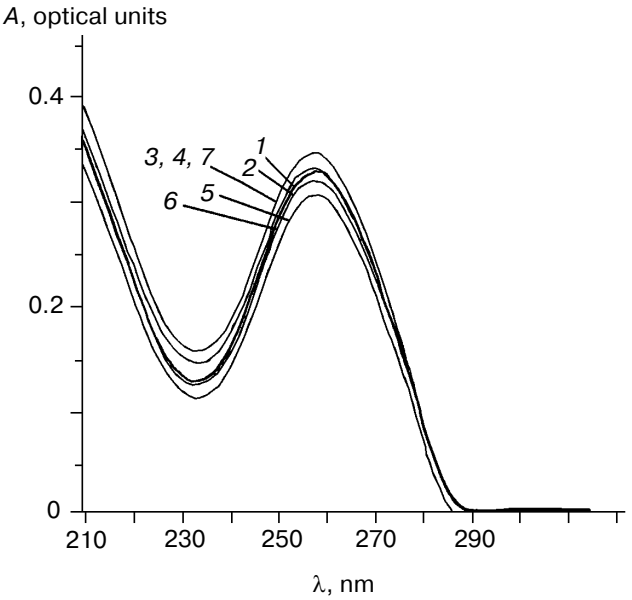

Fig. 2. UV spectra of DNA preparations in the absence (*1*) and in the presence of PCMB: *2-7*) $C_7H_5O_2Hg^+$/nucleotide equivalent ratios vary in the range 3.3-0.000033 corresponding to PCMB concentrations of 0.2 mM-2 nM.

the PCMB concentration (10^4-fold) was followed by both recovery of the enzymatic activity and changing of the PCMB to substrate ratio. However, the enzyme to PCMB ratio was not changed. Therefore, data suggesting a significant role of the substrate in the PCMB-induced change in endonuclease activity were revealed.

The changes in the CD spectra of DNA in the presence of PCMB (Fig. 3) mostly matched the changing endonuclease activity (Fig. 1). Thus, at $C_7H_5O_2Hg^+$/nucleotide equivalent ratio of 3.3-0.33 there was a decrease in the endonuclease activity and in the specific ellipticity in the large positive band of the spectra. At $C_7H_5O_2Hg^+$/nucleotide equivalent ratio of 0.0033-0.0000033, the enzymatic activity and the specific ellipticity increased.

Since the CD spectra serve as a reflection of the secondary structure of the substrate, we showed first that PCMB affects the secondary structure of DNA and second that there is a correlation between the changing secondary structure of the substrate and endonuclease activity. Also, we confirmed the previously published data [10, 11] on the sensitivity of *S. marcescens* endonuclease to the secondary structure of the substrate.

Because the CD spectra of the DNA in the absence of PCMB and at $C_7H_5O_2Hg^+$/nucleotide equivalent ratio of 0.033 were superimposed but the endonuclease activity at that ratio was 25-30% less than the activity in the absence of PCMB (Fig. 3, spectra *1* and *4*; Fig. 1a, [PCMB] = 10^{-4} M), we assume that the *S. marcescens* endonucleases are more sensitive to the change in secondary structure of the substrate than the CD spectra.

Comparing the CD spectra of DNA in the presence of PCMB, $HgCl_2$, and alkali metal ions (Fig. 3, [15]), it is worth commenting on some observations. Although the CD spectra of DNA in the presence of PCMB and $HgCl_2$ were similar in the red shift of the large positive band, they differed in the Cotton effect. Even at $C_7H_5O_2Hg^+$/nucleotide equivalent ratio 0.33-3.3, the Cotton effect drastically differed from the effect in the presence of $HgCl_2$ (Fig. 3, [15, 16]).

By the magnitude of the Cotton effect, the CD spectra measured in the presence of PCMB were like the spec-

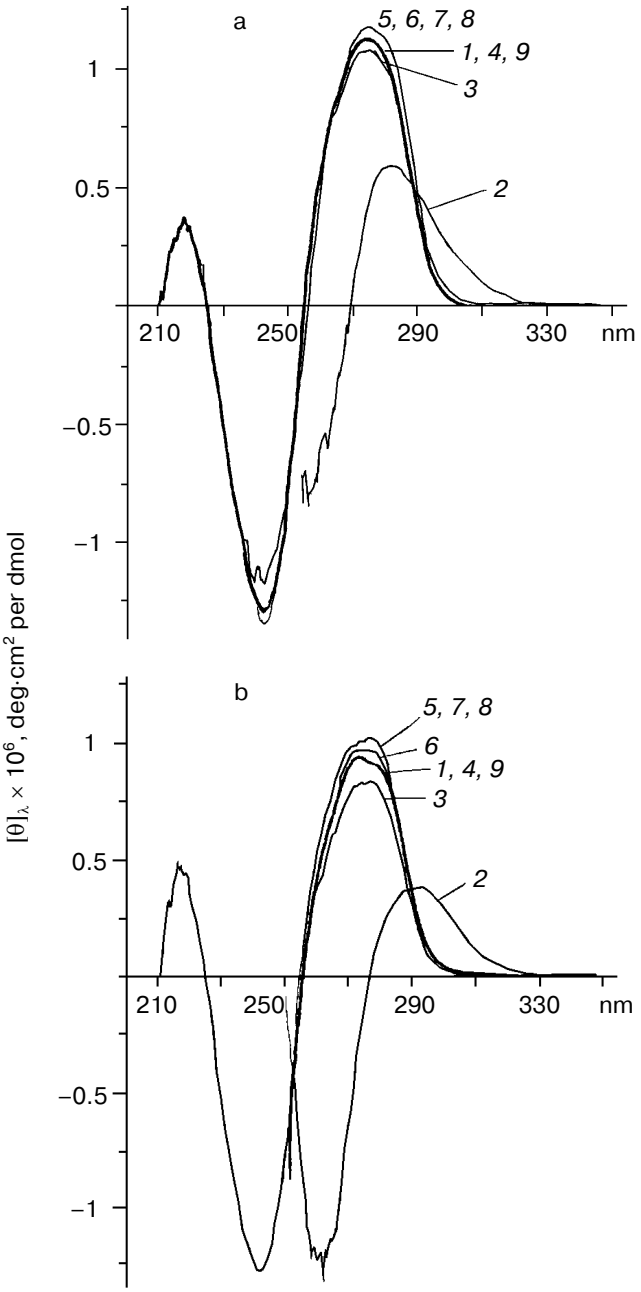

Fig. 3. CD spectra of DNA preparation in the absence (*1*) and in the presence of PCMB: *2-9*) $C_7H_5O_2Hg^+$/nucleotide equivalent ratios vary in the range 3.3-0.00000033 corresponding to PCMB concentrations of 5 mM-0.5 nM in the absence (a) and in the presence (b) of 15 mM Mg^{2+}.

tra of DNA preparations containing Ca^{2+}, Mn^{2+}, or Mg^{2+} [15, 24, 25]. As the weak Cotton effect in the CD spectra of DNA preparations with Ca^{2+}, Mn^{2+}, or Mg^{2+} is associated with their decreased (relative to Hg^{2+}) capacity to react with DNA [15], we suppose $C_7H_5O_2Hg$ cations also have less (compared with Hg^{2+}) ability to react.

The red-shifted center of the large positive band differentiates the CD spectra of DNA containing PCMB from the spectra of DNA measured in the presence of alkali metal cations, in particular Mg^{2+} (Fig. 3, [11]). The red shift is also a distinguishing feature of the CD spectra of Hg^{2+}–DNA compared with the spectra of Mg^{2+}–DNA [14, 15, 24, 25]. From our point of view, this difference may be connected with the difference of Hg^{2+} and Mg^{2+} in their binding sites in polynucleotides. Mg^{2+} is known to form complexes with phosphate groups of DNA, but Hg^{2+} forms bonds with the nitrogen of the bases [13, 14, 20, 24-28]. Thus, the red shift in the CD spectra of DNA measured in the presence of PCMB suggests that $C_7H_5O_2Hg$ cations, like Hg^{2+}, forms complexes with the nitrogen bases of the polynucleotide. The changes in the CD spectra of $C_7H_5O_2Hg$–DNA complexes caused by the addition of Mg^{2+} supports this idea. The addition of Mg^{2+} induces both decreased magnitude of the specific ellipticity in the large positive band that is the characteristic of the effect of Mg^{2+} [11, 24], and the enhancing effect of PCMB increasing the red shift of the large positive band.

Therefore, our analysis showed that PCMB influences *S. marcescens* endonuclease activity by its interaction with the substrate rather than with the enzyme. PCMB forms complexes with DNA, as indicated by the CD spectra, changing the secondary structure of the polynucleotide. These changes influence the enzymatic activity of *S. marcescens* endonuclease (in particular that of isoforms Sm1 and Sm2), which was previously shown to be sensitive to the secondary structure of the substrates [10, 11]. Binding of $C_7H_5O_2Hg^+$ cations to DNA does not form an abortive enzyme–substrate complex; this is shown by the recovery of the PCMB-inactivated *S. marcescens* endonuclease (table). Since Mg^{2+} and $C_7H_5O_2Hg^+$, differing in the metal cation, are similar in their mechanisms of action on the enzymatic activity of *S. marcescens* endonuclease, we expect the mechanism of most other metal-containing effectors towards the endonuclease activity is similar with the elucidated mechanism.

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