tions of heparin in the animal organism, which depend on the physical and chemical properties of its macromolecules [6, 10].

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EFFECT OF THIOL-SPECIFIC REAGENT ON *Pseudomonas aeruginosa* PAEI AND PAEII RESTRICTION ENDONUCLEASE ACTIVITY

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Because of their unique properties class II restriction endonucleases are widely used in research in molecular biology and genetic engineering [2]. By now more than 400 restriction endonucleases have been described, their physicochemical and catalytic properties and the structure of many of these enzymes have been reasonably well studied [7] and techniques have been developed for seeking them in microorganisms and isolating them [3, 4, 6]. However, there is hardly any information in the literature on sulfhydryl groups of restriction endonucleases and their role in interaction with the DNA substrate. The only exception is an investigation [8] of the sensitivity of 11 restriction endonucleases to the action of alkylating and mercury compounds.

The aim of this investigation was to study the importance of SH-groups for manifestation of activity of new restriction endonucleases PaeI and PaeII isolated by the writers from *Pseudomonas aeruginosa*.

EXPERIMENTAL METHOD

Restriction endonucleases PaeI and PaeII were purified from strains of *Ps. aeruginosa* by the method developed by the writers previously [5]. DNA was isolated from phage λ as described previously [1]. The incubation mixture (30-60 µl for determination of the activity of restriction endonucleases PaeI and PaeII contained 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 µg DNA of phage λ, and 1-5 µl of the enzyme preparation. KCl was added to the samples for determination of restriction endonuclease PaeII activity in a final concentration of 20 mM, the samples were incubated at 37°C for 1-2 h, and the reaction was stopped by addition of a mixture containing Na_2-EDTA (pH 8.0), bromphenol blue, and glycerol to final concentrations of 20 mM, 0.02%, and

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5%, respectively. Electrophoretic preparation of the DNA fragments was carried out in 0.8% agarose gel plates as described previously [3]. The minimal quantity of the enzyme (in µl) required to produce complete hydrolysis of 1 µg DNA of phage λ in 1 h under standard conditions was taken as the unit of relative restriction endonuclease activity. The inhibitory action of thiol-specific reagents on restriction endonuclease activity was assessed by a study of the electrophoretic profile of separation of the degradation products of DNA of phage λ. To remove the 2-mercaptoethanol, added in order to stabilize the enzyme for keeping, from the restriction endonuclease preparations gel-filtration was carried out on a column with Sephadex G-50. Thiol-specific reagents — 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB, Ellman's reagents), N-ethylmaleimide (NEM), p-chloromercuribenzoate (PCMB), iodoacetate, and iodoacetamide — were made up in 10 mM glycine solution and the pH adjusted to 8.0 with 1 M NaOH. Reagents for SH-groups were incubated with the restriction endonucleases for 5 min at 37°C, after which phase λ DNA and Mg^{++} ions were added to the samples, which were incubated for 1-2 h.

EXPERIMENTAL RESULTS

On incubation of restriction endonucleases PaeI and PaeII with NEM and DTNB, used in concentrations of 10 mM each, total suppression of activity of these enzymes was found. Meanwhile alkylating (iodoacetate, iodoacetamide, 10 mM of each) and mercury-containing (PCMB, 0.04 or 0.05 mM) compounds had no appreciable inhibitory effect on restriction endonuclease activity (Fig. 1). PCMB inhibited activity of restriction endonuclease PaeI totally or partially in concentrations of 2.5-5 and 1 mM, respectively, but did not change the activity of the enzyme in concentrations of between 0.1 and 0.5 mM. Activity of restriction endonuclease PaeII was suppressed completely by PCMB in concentrations over 0.1 mM. It will be clear from Fig. 2 that total inhibition of restriction endonuclease PaeI activity took place in the presence of DTNB and NEM in concentrations of over 1 and 5 mM, respectively. The minimal concentration of DTNB causing total inhibition of restriction endonuclease PaeII activity was over 0.25 mM, and that of NEM was over 0.5 mM. The results of experiments to study the effect of thiol-specific reagents on activity of restriction endonucleases from *Ps. aeruginosa*, and also data in the literature [8] for enzymes BamHI and SamI (the isoschizomer of restriction endonuclease PaeII) are given in Table 1. According to the sensitivity of the restriction endonucleases to the action of SH-reagents, Nath [8] classified these enzymes into three groups. Activity of restriction endonucleases of the first group (EcoRI, HindIII, SalI, BgIII, HpaI, SstI) was almost unchanged by chemical modification with alkylating and mercury compounds. Enzymes of the second group (BamHI, PvuI, and AvaI) are characterized by participation of SH-groups in the catalytic act. In addition, there is a 3rd group of restriction endonucleases (SmaI, PstI) for whose activity oxidized thiol groups (—S—S—) and not SH-groups are important, since 2-mercaptoethanol potentiated the inhibitory effect of DTNB.

When the above results are analyzed it must be noted that restriction endonucleases PaeI and PaeII correspond approximately in their sensitivity to the inhibitory action of DTNB, NEM, and PCMB to restriction enzymes of the second group (according to Nath's classification): Activity of restriction endonuclease PaeII, moreover, was inhibited in the presence of lower concentrations of the inhibitors than that of PaeI. Another matter worthy of attention is that 2-mercaptoethanol did not potentiate the inhibitory effect of DTNB and NEM on restriction endo-

TABLE 1. Effect of Thiol-Specific Reagents on Activity of Restriction Endonucleases from *Ps. aeruginosa*

Enzyme	Reagents for SH-groups		
	DENP	NEM	PCMB
PaeI	>1	>5	>1
PaeII	>0,25	>0,5	>0,1
SmaI	>10	>167	>1,6
BamHI	>5	—	>1,8

Legend. Concentrations of reagents inhibiting restriction endonuclease activity by 100% are given (in mM). Data for restriction endonucleases SamI and BamHI taken from [8].

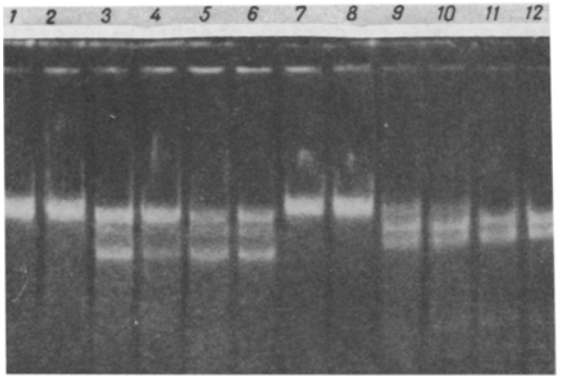

Fig. 1

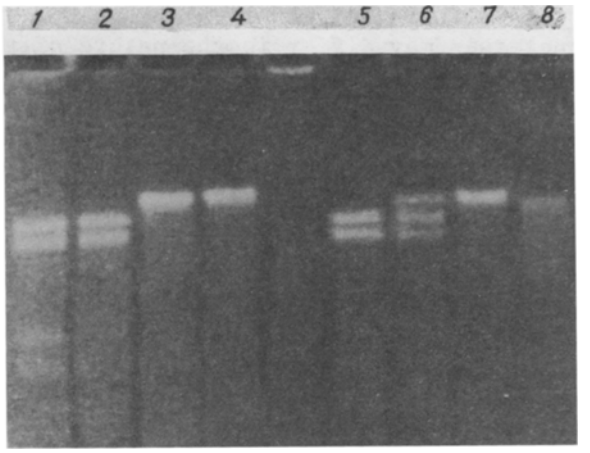

Fig. 2

Fig. 1. Effect of inhibitors of SH-groups on activity of restriction endonucleases PaeI (7-12) and PaeII (1-6). 1 and 7) NEM (10 mM); 2 and 8) PTMB (10 mM); 3 and 9) iodoacetate (10 mM); 4 and 10) iodoacetamide (10 mM); 5 and 11, 6 and 12) PCMB (0.01 and 0.05 mM, respectively).

Fig. 2. Inhibition of activity of restriction endonuclease PaeI by the action of DTNB and NEM. 1-4) NEM in concentrations of 1, 2.5, 5, and 7.5 mM, respectively; 5-8) DTNB in concentrations of 0.1, 0.5, 1, and 2.5 mM, respectively.

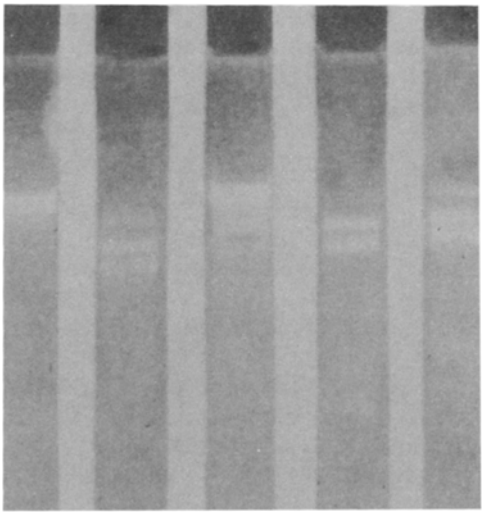

Fig. 3. Effect of DNA on phage λ, Mg^{++} ions, and 2-mercaptoethanol on inhibitory activity of restriction endonuclease PaeI on effect of DTNB. 1) PaeI + DTNB (1 mM); 2) enzyme incubated with DNA of phage λ; 3) with Mg^{++}; 4) with DNA of phage λ + Mg^{++}; I) with 2-mercaptoethanol. After preincubation (5 min, 37°C) DTNB (1 mM) was added to the samples and incubation continued for 1 h.

nuclease PaeII activity. Meanwhile the reducing SH-groups of the compound potentiate inhibition of activity of restriction endonuclease SmaI by DTNB [8]. The writers showed previously that the PaeII and SmaI enzymes are true isoschizomers, i.e., they recognize the same hexanucleotide sequence of DNA (5'-CCC↓GGG-3') and cut it at the same place [5]. Thus differences in the sensitivity of restriction endonucleases PaeII and SmaI to the inhibitory effect of DTNB, NEM, and PCMB, and also the different effect of 2-mercaptoethanol on inhibition of the

activity of these enzymes by DTNB and NEM are evidence that the isoschizomers of restriction endonucleases may differ in the nature of their functional groups responsible for binding and (or) conversion of the DNA substrate. We know that substrates, their analogs, and coenzymes can protect the SH-groups of enzymes against the inhibitory action of thiol-specific reagents, indicating the importance of SH-groups for substrate binding. So far as the restriction endonucleases are concerned, there are no data on this subject in the literature.

Electrophoretic separation of the products of hydrolysis of phage λ DNA by restriction endonuclease PaeI in experiments to study the protective action of the DNA substrate, Mg^{++} ions, and 2-mercaptoethanol on the inhibitory effect of DTNB is shown in Fig. 3. Restriction endonuclease PaeI was incubated for 5 min at 37°C with phage λ DNA (1 μg), with Mg^{++} ions (10 mM), with phage λ DNA + Mg^{++}, or with 2-mercaptoethanol (80 mM), after which DTNB was added to the samples in a concentration of 1 mM, which completely suppressed activity of the enzyme, and incubation continued for a further 1 h. It was found that phage λ DNA had a marked protective action against inhibition of restriction endonuclease PaeI activity by DTNB (lane 2), whereas Mg^{++} ions prevented the decrease in enzyme activity caused by DTNB only weakly (lane 3). On simultaneous addition of phage λ DNA and Mg^{++} ions to the incubation mixture total abolition of the inhibitory action of DTMB on restriction endonuclease PaeI activity was observed (lane 4). Approximately the same results were obtained for restriction endonuclease PaeII. Incidentally, 2-mercaptoethanol partly prevented the blocking effect of DTNB.

The results of these experiments show that SH-groups essential for activity of the enzymes and, probably, responsible for binding of the restriction endonucleases with the DNA substrate, are present in the structure of restriction endonucleases PaeI and PaeII. However, the possibility likewise cannot be ruled out that the SH-groups may help to maintain the structure of the enzyme molecule in a stable, catalytically active state.

The results showing the effect of thiol-specific reagents on activity of restriction endonucleases PaeI and PaeII also have a purely practical significance, associated with the fact that reducing agents, such as 2-mercaptoethanol or dithiothreitol, must be present in the buffered solution in order to stabilize restriction endonucleases PaeI and PaeII during their isolation, storage, and assay.

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