2-Methyl-4-carboxy,5-hydroxy-3,4,5,6-tetrahydropyrimidinidom (THP(A) or hydroxyectoine) and 2-methyl-4-carboxy-3,4,5,6-tetrahydropyrimidinidom (THP(B) or ectoine) are now recognized as ubiquitous bacterial osmoprotectants. To evaluate the impact of tetrahydropyrimidinidom derivatives (THPs) on protein-DNA interaction and on restriction-modification systems, we have examined their effect on the cleavage of plasmid DNA by 10 type II restriction endonucleases. THP(A) completely arrested the cleavage of plasmid and bacteriophage λ DNA by EcoRI endonuclease at 0.4 mM and the oligonucleotide (d(CCGGAATTCCGCG))2 at about 4.0 mM. THP(B) was 10-fold less effective than THP(A), whereas for betaine and proline, a notable inhibition was observed only at 100 mM. Similar effects of THP(A) were observed for all tested restriction endonucleases, except for SmaI and PvuII, which were inhibited only partially at 50 mM THP(A). No effect of THP(A) on the activity of DNase I, RNase A, and Taq DNA polymerase was noticed. Gel-shift assays showed that THP(A) inhibited the EcoRI-(d(CCGGAATTCCGCG))2 complex formation, whereas facilitated diffusion of EcoRI along the DNA was not affected. Methylation of the carboxy group significantly decreased the activity of THPs, suggesting that their zwitterionic character is essential for the inhibition effect. Possible mechanisms of inhibition, the role of THPs in the modulation of the protein-DNA interaction, and the in vivo relevance of the observed phenomena are discussed.

Two tetrahydropyrimidinidom derivatives identified in Streptomyces bacteria (1–3), one a previously known metabolite, THP(A), and the other previously identified (as ectoine) in halophilic bacteria (4), THP(B), are now recognized as widely spread osmoprotectants within the bacterial world (5). The role and activities of THPs are of special interest as they represent a limited group of osmoprotectants that are synthesized de novo, in the bacterial cell, in contrast to those transported from the medium (6). Their synthesis in a number of Streptomyces strains as a response to increased salinity and elevated temperature was recently described (7). THPs are small molecules, highly soluble in water and neutral at physiological pH. NMR and x-ray crystallography data show that THPs are zwitterionic molecules with a delocalized π charge in the NCN group (Fig. 1) and form the half-chair conformation (8).

More information has been accumulated lately on THPs activity in living cells. It was found that exogenously provided ectoine (THP(B)) could reverse growth inhibition, caused by osmotic stress, in Escherichia coli (9), Corynebacterium glutamicum (10), and the soil bacterium Rhizobium meliloti (11). We demonstrated that exogenously provided THP(A), like THP(B), reversed inhibition of E. coli growth by osmotic stress, and moreover, both THP(A) and THP(B) could stimulate growth of E. coli at an elevated temperature (43 °C) (7). Recently cloned genes for ectoine synthesis from Halomonas elongata (12) and from Marinococcus halophilus (13) were demonstrated to be both necessary for halotolerance of ectoine-producing bacteria and osmotically regulated.

The ability of osmoprotectants (e.g. proline and betaine) to overcome the inhibitory effect of osmotic stress in bacteria was traditionally explained in two ways. One hypothesis states that proline and betaine have special interactions with proteins that protect them from denaturation in the presence of high concentrations of electrolytes (14, 15). In support of this mechanism, it was recently shown that THPs stabilize proteins upon freezing and at elevated temperatures (16, 17). According to another hypothesis, proline and betaine are merely aimed at maintaining cell turgor in media of high osmolality, being compatible with normal cellular functions at high intracellular concentration. Intracellular concentrations of THPs attain up to 158 mM in nonhalophilic Streptomyces bacteria (7), and as high as 2.25 M in the halophilic bacterium H. elongata (18), whereas betaine can be synthesized to 0.6 M in the Methanohalophilus strain Z7401 (19) and proline to 0.7 M in Bacillus subtilis (20). At these high concentrations, proline, betaine, and THP(B) demonstrate a pronounced destabilizing effect on DNA in vitro (22–24). Moreover, THP(B) at certain concentrations, can, like betaine (22), eliminate base pair composition dependence of DNA melting. In addition, THP molecules share similarity in structure and geometry with pyrimidine bases and have the zwitterionic character, which was recently shown to be responsible for modification of DNA electrostatic interaction with counterions by a number of osmolytes (25). These notions led us to suggest that the impact of osmoprotectants, and THPs in particular, on protein-DNA interaction is, likely, underestimated. The destabilization of the DNA duplex could play a dual role in the interaction of DNA with proteins; it...
may either enhance binding, as it was shown for proline and SSB protein, or diminish it, as it was shown for DNaseI, for which double-stranded DNA serves as a substrate (24). Yet the impact of osmolytes on the sequence-specific interaction of proteins with DNA (e.g., restriction endonucleases and transcription factors) could be even more complex and remains to be elucidated.

Type II restriction enzymes are recognized as an ideal model system for evaluating site-specific protein-DNA interaction, as any interference with the precise alignment of the enzyme and substrate will affect the cleavage. It has been demonstrated that the binding of ligands, such as antibiotics and dyes, reduces the activity and specificity of restriction endonucleases (26). It has also been shown that the constituents of bacterial cells, such as polyamines and basic proteins (27) or polyphosphate (28), are also capable of inhibiting the DNA cleavage by restriction endonucleases in vitro. Hence, the primary function of the restriction-modification system, aimed at protecting bacteria from phage infection or from other sources of foreign DNA, might be dependent on the composition of intracellular metabolites.

In the present study, we have investigated the effect of bacterial osmolytes, such as THP(A), THP(B), proline, and glycine betaine, on DNA cleavage by several type II restriction endonucleases, taken as a model system for specific protein-DNA interaction. The results were compared with the effect on proteins that bind nucleic acid nonspecifically, such as DNaseI, RNase A, and Taq DNA polymerase. We report here that THP(A) and, to a lesser extent, THP(B) are capable of completely arresting the DNA cleavage by a number of restriction endonucleases, whereas proline and glycine betaine are almost ineffective. The effect of THPs on EcoRI binding to the oligonucleotide, containing its recognition site, and on linear diffusion was also examined.

A possible role of THPs in the modulation of the restriction-modification system and in gene expression under stress is suggested.

MATERIALS AND METHODS

Plasmids pGE1M1 and pBR322 were isolated from E. coli strain DH5α by alkaline lysis and purified by equilibrium centrifugation in a gradient of cesium chloride (29). Restriction endonuclease EcoRI, PsvII, AvaI, and DraI were purchased from New England Biolabs, SspI and SmaI were from MBI Fermentas, and EcoRV were from Boehringer Mannheim. Pancreatic bovine deoxyribonuclease I, ribonuclease A, yeast RNA, betaine monohydrate (glycine betaine), and spermine tetrahydrochloride were from Sigma, l-proline (99.5%) was from Fluka, and Thermus aquaticus DNA polymerase (recombinant) was from MBI Fermentas.

Preparation of Tetrahydroprimidinyl Derivatives—THPs were prepared and purified as described previously (7). The activity of THPs preparations in the restriction assay was checked before and after passing through a Chelex column, and no difference was observed. THPs preparations were further analyzed by atomic flame photometry, and no detectable amounts of Mg, Ca, and Fe ions and less than 0.1% sodium and potassium ions were found. The micromolar final concentration of Na+ or K+ upon addition of THPs to the reaction mixture is negligible in comparison to 50–100 mM potassium in the buffer. For the preparation of 2-methyl, 4-methylcarboxylate, 5-hydroxy-3,4,5,6-tetrahydroprimidinyl (methyl-THP(A)) and 2-methyl, 4-methylcarboxylate-3,4,5,6-tetrahydroprimidinyl (methyl-THP(B)) (Fig. 1), an excess of thionolymidyl was gradually added to a 0.5 mM solution of THP(A) or THP(B) in methanol at 0°C under continuous stirring. Resulting solutions were evaporated under reduced pressure and the residue, containing CH₃-THP(A)/HCl or CH₃-THP(B)/HCl, was dissolved in water and neutralized by NaOH to pH 7.0. Water was evaporated under reduced pressure, and the solid material was resuspended in methanol, separated from NaCl by filtration, and dried. The purity of methyl-THP(A) and methyl-THP(B) was determined by 1H and 13C NMR (data not shown).

Preparation of the Labeled Oligonucleotide—(dCGCGAATTCGCG)₂ was prepared by solid phase synthesis (Chemical Services, Weizmann Institute of Science), purified on a denaturing polyacrylamide gel containing 7 M urea, and desalted on Sephadex G-25. The oligonucleotide was labeled with [γ-³²P]ATP (6000 Ci/mmol, New England Biolabs) by T4 polynucleotide kinase (30), purified by denaturing polyacrylamide gel, and desalted on Sephadex G-25. The ³²P-labeled oligonucleotide was heated for 5 min at 90 °C in a buffer containing 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM MgCl₂, 0.1 mM dithiothreitol and then slowly cooled to 4 °C, desalted on Sephadex G-25, purified by step elution on DEAE-Sephadex (10 mM bis-Tris propane, pH 7.4), with increasing concentrations of KCl (up to 0.6 M KCl) and desalted by dialysis against water. About 95% of the resulting oligonucleotide was cleavable by EcoRI endonuclease.

Cleavage by Restriction Endonucleases—Cleavage of DNA by each restriction enzyme was carried out in a total volume of 20 μl of the reaction buffer recommended by manufacturer. The cleavage reaction was initiated by addition of the enzyme to the incubation mixture containing THPs or other additives and terminated by addition of EDTA to a final concentration of 20 mM. Reaction products were analyzed by electrophoresis on a horizontal 1% agarose gel at 3 V/cm. Gels were stained with ethidium bromide and photographed, and pictures were quantified by densitometry.

Cleavage of dodecadeoxynucleotide by EcoRI was carried out in 20 μl of buffer containing 50 mM NaCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM dithiothreitol, 10 mM MgCl₂, and 50 mg/ml bovine serum albumin. Products of the ³²P-labeled dodecadeoxynucleotide cleavage were analyzed by electrophoresis on a 20% polyacrylamide gel containing 7.0 M urea, visualized by autoradiography and quantified by densitometry.

DNase I, RNase A, and Taq Polymerase Assays—The DNase I assay was performed at 37 °C in 100 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 100 mM NaCl. A minimal amount of enzyme (0.3 ng/ml) needed for complete transformation of supercoiled plasmid DNA into nicked circular within 1 h (as determined in a separate experiment in the absence of THPs) was added to the reaction mixture containing THPs. Aliquots, taken after short intervals of incubation (every 5 or 10 min), were immediately transferred into liquid nitrogen and kept frozen until loaded on the agarose gel.

RNase A assay was carried out at 37 °C in 20 μl of buffer containing 1 μg of yeast RNA, 20 mM Tris-HCl, pH 7.2, 50 mM NaCl, and 2 mM MgCl₂. The minimal amount of enzyme needed for 50% digest of RNA within 30 min (0.2 μg/ml, as determined in a separate experiment in the
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FIG. 2. Cleavage of pBR322 DNA by EcoRI endonuclease in the presence of THP(A). A, pBR322 DNA (25 μg/ml) was incubated with 125 units/ml EcoRI in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM NaCl in the absence and presence of THP(A), as indicated. Reaction products were analyzed on a 1% agarose gel. The positions of supercoiled (sc), linear (li), and nicked (ni) DNA are indicated. B, Dixon plot of pBR322 DNA cleavage by EcoRI in the presence of THP(A). The data points for each DNA concentration are the average of at least three independent kinetic experiments. ■, 2 μg pBR322 DNA; ○, 4 μg; ▲, 6 μg. C, Cornish-Bowden plot of the data presented in B. ■, 2 μg pBR322 DNA; ○, 4 μg; ▲, 6 μg.

FIG. 3. Effect of osmolytes and methyl derivatives of THPs on the rate of pBR322 DNA cleavage by EcoRI. Every data point reflects the initial rate of cleavage (the region of linear reaction kinetics has been determined in a separate experiment) of pBR322 DNA (37 μg/ml) by EcoRI restriction endonuclease (37 units/ml) in the presence of the corresponding solute, normalized by the rate of cleavage in the absence of additives. ■, THP(A); ○, THP(B); □, methyl-THP(A); ○, methyl-THP(B); △, l-proline; ▲, glycine betaine. Data points are the average of at least two independent experiments.

as determined in a separate experiment). The reaction was stopped by addition of 10 μl of 50 mM EDTA and applied to strips of chromatographic paper (Whatman No. 3). Strips were washed three times with ice-cold trichloroacetic acid and dried, and the radioactivity was measured by a scintillation counter.

Gel-shift Assay—Assays on EcoRI binding to the dodecéadexynucleotide were carried out in 20 μl of binding buffer containing 50 mM NaCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 50 μg/ml bovine serum albumin. THPs were added to the reaction mixture and incubated at room temperature for 15 min before or after the addition of EcoRI, 8 μl of the loading buffer (40% w/v sucrose and 100 μg/ml bromphenol blue) was added, and the samples were loaded on the 9% (29:1, acrylamide:bisacrylamide) polyacrylamide gel, 45 mM Tris borate (pH 8.0), and 2.0 mM EDTA. Gels were prerun for 2–3 h prior to loading of samples and after electrophoresis for 3–4 h at 10–12 V/cm and 4 °C, dried, and autoradiographed, and the resulting negatives were subjected to densitometry.

Effect of THPs on the Linear Diffusion of Restriction Endonucleases—We have basically followed the experiment developed by Ehrbrecht et al. (31). To evaluate the contribution of linear diffusion to the rate of the EcoRI-catalyzed reaction, the difference in the cleavage rate of short (378 bp) and long (4361 bp) fragments of pBR322 plasmid DNA with a centrally located target site by EcoRI endonuclease (see Fig. 5) was measured. To prepare short and long DNA fragments, 300 μg of pBR322 plasmid DNA was cleaved overnight by SspI/EcoRV or PvuII endonucleases (60 units each) in the buffers recommended by the manufacturers. The resulting digest mixture was used without further purification as a substrate for EcoRI restriction endonuclease.

As was shown previously, the components of the primary reaction do not affect the secondary reaction (31). This experimental setup ensures that factors of the reaction other than the length of the substrate are invariant; namely, all fragments with the EcoRI site have identical flanking sequences, and due to the presence of fragments not containing the EcoRI site, nonspecific binding is identical in all experiments. The secondary reaction (with EcoRI endonuclease) was carried out in the optimized buffer (31) containing 20 mM Tris-HCl, pH 7.2, 0.05 mM MgCl₂ bovine serum albumin, 50 mM NaCl, and 1 mM MgCl₂ in the presence of varying amounts of THPs.
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RESULTS

Effect of THPs and Osmolytes on DNA Cleavage by EcoRI Endonuclease—We have studied the effect of THP(A), THP(B), proline, and betaine on plasmid DNA cleavage by EcoRI endonuclease. The rate of cleavage of pBR322 DNA by EcoRI endonuclease was notably decreased, starting from 0.1 mM THP(A). It is worth noting that no intermediate products such as open circular DNA were accumulated upon inhibition (Fig. 2A). To determine the type of inhibition and estimate the inhibition constant, the steady state kinetics of cleavage was measured at constant DNA concentrations and varying concentrations of THP(A). A Dixon plot (32) of these data (Fig. 2B) with $V_{\text{max}}$ constant within the range of error, combined with a set of parallel lines in a Cornish-Bowden plot (33) (Fig. 2C), indicates a competitive inhibition (34) with $K_i = 0.16 \pm 0.04$ mM. The inhibition effect of other osmolytes was much less pronounced; i.e. whereas THP(A) completely arrested plasmid DNA cleavage at around 1.0 mM, THP(B) was 10-fold less effective, betaine and proline at low concentrations showed stimulation of DNA cleavage, and a notable inhibition was observed only at 100 mM (Fig. 3). It is worth noting that at this high concentration, additional effects, such as a decrease of water activity, a significant increase of the medium dielectric constant, or changes in the pH of the reaction mixture, are possible. The significant increase of the medium dielectric constant, or influence decreasing the lifetime of the nonspecifically bound protein (totally electrostatic binding mode) along the DNA, is thought to occur by the displacement of bound (delocalized) positive counterions from the DNA (36). Thus, any localized) positive counterions from the DNA (36). Thus, any localized positive counterions from the DNA (36). Thus, any localized positive counterions from the DNA (36). Thus, any localized positive counterions from the DNA (36). Thus, any localized positive counterions from the DNA (36).

The cleavage of dodecadecoxynucleotide duplex by EcoRI was tested in the presence of THP(A) with the minimal amount of enzyme needed for complete cleavage and with a 5-fold excess of the enzyme. Similar results were observed in both cases (Fig. 4A, and B). The complete (95–100%) inhibition of the reaction was reached at 5 mM THP(A). The higher concentration of THP(A) needed for complete inhibition of the oligonucleotide cleavage (3–5-fold) as compared with plasmid DNA could be attributed to the different reaction conditions employed in the two experiments, different flanking sequence of recognition sites, and known differences in the reaction kinetics of restriction enzymes on short duplexes of 8–12 bp compared with those on longer DNA (35).

The inhibition of EcoRI endonuclease reaction by THP(A) was not compensated by increased concentration of MgCl$_2$ (20 mM instead of 10 mM) in the reaction mixture, suggesting that sequestering of Mg$^{2+}$ ions is not responsible for the observed effect. In both cases, the inhibition of DNA cleavage by THP(A) was similar to that depicted in Fig. 3.

Do THPs Affect the Linear Diffusion of Endonuclease along the DNA?—The cleavage of the plasmid DNA substrate by restriction endonucleases is preceded by a number of consecutive events, such as a nonspecific association with the DNA and linear diffusion of the nonspecifically bound protein along the DNA until the recognition site is reached and specific binding occurs. On exploring the possible targets for THP(A) action, we further investigated its effect on the linear diffusion of EcoRI along the DNA by using the experimental approach developed by Ehbrecht et al. (31). To ensure that the difference in the rate of cleavage in this experiment originates solely from the chain length of the substrate, the plasmid DNA (bearing a single EcoRI site) was cleaved beforehand by other restriction endonucleases, producing two sets of DNA fragments of different length. The resulting mixture was used as a substrate for EcoRI endonuclease, thus excluding possible effect of different flanking sequences and equalizing the nonspecific binding. Plasmid pBR322 was cleaved by PvuII endonuclease, to yield a linear fragment of 4361 bp. Alternatively, plasmid DNA was cleaved by EcoRI/SspI, resulting in a 3983-bp fragment without a recognition site and a 378-bp fragment with a centrally located EcoRI site (Figs. 5 and 6A). Using optimized concentration of Mg$^{2+}$ (31) we observed a distinct difference in the cleavage rate of the long and short DNA fragments by EcoRI endonuclease, ranging from 20 to 8% (Fig. 6, A and B). This enhancement of the reaction rate, proportional to the length of the DNA substrate, is usually explained in terms of sliding or intersegment transfer (36). The sliding process can be viewed as a one-dimensional diffusion of the nonspecifically bound protein (totally electrostatic binding mode) along the DNA, which is thought to occur by the displacement of bound (delocalized) positive counterions from the DNA (36). Thus, any influence decreasing the lifetime of the nonspecifically bound state (e.g. increase of salt concentration (37)) or presenting a steric obstacle to linear movement (e.g. nonsaturating amounts
of histone-like protein Hu (31)) will notably diminish the reaction rate dependence on the DNA length. This was not the case for the inhibition of the EcoRI cleavage reaction by THPs. On the contrary, we found that the difference in the cleavage rate of the long and short DNA fragments somewhat increased upon increasing THPs concentrations (Fig. 6), suggesting that the linear diffusion was not significantly affected by THPs in the concentration range employed. Higher concentrations of THP(A) led to very low levels of remaining endonuclease activity for short DNA fragments to be quantified from a gel and compared with the rate of cleavage of long DNA fragments.

THP(A) Affects EcoRI Binding to DNA—It is known that EcoRI endonuclease first binds to its recognition site and then the enzyme-DNA complex binds Mg²⁺ (38). Thus, the DNA-protein complex formation in the absence of Mg²⁺ can be used to probe the effect of THP(A) on EcoRI binding to DNA. The experiments were conducted with a minimal amount of EcoRI endonuclease, needed for complete binding of the d(CGCGAATTCTCGCG) duplex, and with a 5-fold excess of the enzyme, and they exhibited similar results. As shown in Fig. 7, A and B, THP(A) inhibited EcoRI-oligonucleotide complex formation, starting at 1–1.5 mM concentrations, with an inhibition constant, Kᵢ (50% inhibition of binding), of 2.0–2.5 mM. Higher THP(A) concentrations, 3.5–4.0 mM, provided complete (95–100%) inhibition. These data indicate that THP(A) arrested the DNA cleavage by EcoRI as soon as the first step of the reaction by preventing EcoRI binding to its target DNA sequence.

Effect of Methylated Derivatives of THPs—In order to establish whether the zwitterionic nature of THPs is necessary for the observed inhibition effect, we prepared carboxymethyl derivatives of THPs (Fig. 1) and tested their effect on the reaction catalyzed by EcoRI endonuclease. The ability of these compounds to inhibit the endonuclease reaction was almost 100-fold lower, in comparison to the respective THPs, and at low concentrations, the methylated THP(B) derivative even enhanced the reaction rate as seen in Fig. 3 (note the logarithmic scale). It is possible that the slight inhibition effect was observed due to the minor amounts of unmethylated THPs remaining in the preparation.

Effect of THP(A) on Several Type II Restriction Endonucleases—The inhibition of DNA cleavage (95–100%) by THP(A) was also demonstrated for other type II restriction endonucleases (Table I), in the range of 2–4 mM. Although the incubation buffers were different for most of the enzymes used, we have not noticed any correlation of the observed inhibition effect to the buffer composition. The finding that THP(A) inhibits SgrAI restriction endonuclease was unexpected, because both SgrAI and THP(A) originate from the same microorganism Streptomyces griseus (7). However, a few endonucleases appeared to be less sensitive to THP(A), e.g., more than 90% of the remaining activity was detected at 50 mM THP(A) for SmaI endonuclease and about 53% for PvuII. As SmaI is known to be a thermosensitive enzyme, experiments with this endonuclease were conducted at 30, 28, and 24 °C and showed similar results. To verify whether a temperature lower than that of 37 °C, commonly used throughout this study, can diminish the effect of THP(A), the assay for EcoRI endonuclease was also conducted at 30 °C, with an effect similar to that at 37 °C.

To assess whether the inhibition by THP(A) is dependent on the nature of recognition sites, we further examined the effect of THP(A) on the cleavage of λ phage DNA by AvaI restriction endonuclease. AvaI has eight recognition sites on λ phage DNA, CCCGGG (three sites), CCCGAG (two sites), CTCCGG (two sites), and one site of the CTCCGAG sequence. It appeared that not all of these sites were equally sensitive to the inhibition by THP(A). The appearance of the band of 6500 bp (Fig. 8) may indicate the preferential inhibition of the cleavage of the CTCCGAG recognition site of AvaI. Alternatively, this observation may reflect that AvaI cleaves CTCCGAG with the lowest rate as compared with the other seven sites, although such a hierarchy of sites was not observed previously for this enzyme (39). It is worth noting that this sequence is characterized by the lowest GC content among the eight others, whereas the highest GC content has the sequence identical to the recognition site of SmaI (Table I), although SmaI produces DNA fragments with blunt ends, comparing to overhang ends for the AvaI.

DNase I, RNase A, and Taq Polymerase Reactions Are Not Affected by THPs—For the sake of comparison to endonucleases, we examined the effect of THPs on the reaction catalyzed by DNase I, RNase A, and Taq polymerase, enzymes interacting with nucleic acids in a nonspecific manner. All three enzymes were extensively studied, and the structures of their complexes with nucleic acids have been solved (40–42). THP(A), THP(B), or the mixture of THP(A) and THP(B), as it frequently appears in the microbial cell, up to 27 mM did not affect the rate of the DNase I reaction. In addition, no changes in the reactions catalyzed by RNase A or Taq polymerase were observed in the presence of up to 60 and 500 mM THPs, respectively.

Spermine Restores DNA Cleavage and Binding by EcoRI, Arrested by Moderate THP(A) Concentrations—The finding that even the low level of THP(A) in S. griseus at normal physiological conditions is sufficient to inactivate SgrAI endonuclease in vitro raised the question of how the restriction system functions in bacteria that produce or accumulate this osmolyte. It is well known that DNA in vivo is complexed with polyamines, which are essential components of many, if not all, prokaryotic organisms (for a review, see Ref. 43). In E. coli, concentrations of putrescine and spermidine range from 64 to 8 and from 6 to 18 mmol/liter of cytoplasmic water, respectively, depending on the medium osmolarity (44). In mesophile Streptomyces bacteria (including S. griseus), in addition to putrescine and spermidine, spermine is commonly detected (45). To assess whether polyamines could be remedial for the restriction system inhibited by THPs, we studied the effect of spermine on EcoRI binding to and cleavage of [(dCGCGAATTCTCGG)]₂ oligonucleotide, arrested by THP(A). We found that spermine
from a concentration of 0.1 mM starts to restore DNA cleavage inhibited by 5 mM THP(A), whereas at 3 mM, it already inhibits the cleavage by itself (Fig. 9A). Fig. 9B shows that spermine restores EcoRI binding to oligonucleotide, starting from 0.1 mM, and leads to complete binding at 0.5 mM. Nevertheless, as seen in the Fig. 9B, spermine only partially restores EcoRI binding to oligonucleotide arrested by 10 mM THP(A).

**DISCUSSION**

Possible Mechanism(s) of THP Effect—To elucidate the molecular basis of the inhibition effect of THPs, the impact of these compounds on every element of the endonuclease reaction has to be considered. Our results reveal that THPs do not denature or modify the restriction enzymes in the reaction mixture. This notion is also supported by the findings that THPs can preserve activity of different enzymes (46, 17). The possibility that THPs act by sequestering magnesium ions in the reaction mixture can be ruled out. Furthermore, THP(A) inhibits binding of EcoRI to DNA, for which Mg$^{2+}$ is not required. Our kinetics data indicate a competitive type of inhibition; with $V_{\text{max}}$ remaining constant and estimated $K_i = 0.16 \pm 0.04$ mM. This type of inhibition suggests that the inhibitor solely interferes with the enzyme-substrate complex formation, without affecting the catalytic step of the reaction. This notion was further supported by gel-shift analysis, which revealed inhibition of EcoRI oligonucleotide binding by THP(A) in millimolar concentrations. A few mechanisms for THPs interference with enzyme-DNA association are possible. The inhibitor can displace the substrate from the active site of the enzyme, or recombine with the substrate, or modulate energetic components of binding without direct interaction with the enzyme or DNA. Direct binding of THP(A) to DNA is unlikely, as was revealed by equilibrium dialysis and the absence of effect on DNA melting. In contrast, a very weak interaction with DNA was shown by a two-dimensional NMR study for THP(B), along with lowering DNA melting temperature and elimination of the dependence of DNA melting on base pair composition. Yet THP(B) destabilized the DNA duplex at much higher concentrations (1–4 mM) than that needed to inhibit the endonuclease reaction and was 10-fold less effective in inhibiting endonucleases than THP(A), suggesting that effects other than DNA destabilization are responsible for endonuclease inhibition by THPs.

To ensure fast location and binding to a specific site on DNA, restriction endonucleases first bind nonspecifically anywhere to the DNA and then scan the DNA in search of its recognition site in a one-dimensional diffusion process (47, 48). The existence of sliding as a mean of target location for DNA-binding proteins has been described also in a number of other systems, such as transcription regulatory proteins and RNA polymerase binding to its promoter site (36). In an attempt to assess whether the steps of the enzyme association with DNA can be inhibited independently or cooperatively by THP(A), we have found that the linear diffusion of EcoRI, and consequently a nonspecific binding as a prerequisite of linear diffusion, was unaffected up to 1.5 mM THP(A) (Fig. 6, B and C). Linear diffusion is dependent mostly on the electrostatic interactions between the protein and the phosphate groups of the DNA (47). Because of its electrostatic nature, nonspecific binding is known to be strongly dependent on the salt concentration of the buffer. THPs most likely do not distort the electrostatic equilibrium to the same extent as do inorganic ions, which makes them preferable to salts as intracellular osmolytes.

Insensitivity of enzymatic reactions catalyzed by proteins that bind DNA nonspecifically, such as DNase I, RNase A, and Taq DNA polymerase, to inhibition by THPs supports the above notion that these compounds are “safe” to nonspecific
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Fig. 7. Inhibition of EcoRI-(d(CCGGAATTCCGCG))₆ complex formation by THP(A). A, electrophoretic mobility of EcoRI-dodecanucleotide complexes in the presence of increasing concentrations of THP(A) in nondenaturing polyacrylamide gel. (d(CCGGAATTCCGCG))₆ (0.1 mM) was incubated with 5 units of EcoRI in 20 µl of binding buffer; B, inhibition curve, calculated as a ratio of bound to total DNA, obtained from the densitometry of the polyacrylamide gel autoradiograph presented in A. The enzyme-DNA complex formation was followed by electrophoresis as described under “Materials and Methods.”

protein-DNA interactions. It is important to note that both DNase I (49) and RNase A (42) are characterized by extensive interaction with the DNA phosphate backbone.

We further compared the effect of THPs within a number of type II restriction endonuclease that differ in sequence of recognition site, GC/AT ratio, cleavage pattern (blunt or overhang ends), etc. (Table I). The observation that one out of three recognition patterns of Avai endonuclease on λ DNA, was preferentially inhibited by THP(A) suggested that inhibition may be sequence-dependent. Another striking observation was that PvuII and SmaI endonucleases were much less sensitive to THPs inhibition effect than all other restriction enzymes studied. What are the features of SmaI and PvuII interaction with DNA that make them respond differently than other restriction endonucleases tested for inhibition by THPs? Withers and Dunbar (50) noticed that PvuII displays certain similarities with SmaI, such as (i) interaction with each of the base pairs within the recognition site, (ii) a potential protein contact to the phosphate 3' to the scissile bond, and (iii) no significant bending of the DNA. In addition, SmaI interactions with DNA phosphates are all adjacent and clustered within the recognition site (50), whereas EcoRV endonuclease exhibit phosphate interactions both within and flanking the recognition sequence (51). In EcoRI, DNA phosphate interactions are delocalized, so the primary clamp occurs at the immediate 5'-end of the recognition sequence, and supplementary clamps occur adjacent to the primary and at the center of the recognition sequence (52). The most remarkable common feature of SmaI and PvuII is that the PvuII does not distort DNA while bound to its recognition sequence (53), and SmaI bends DNA insignificantly (50). This distinguishes them from the other well studied endonucleases, e.g., upon binding to canonical DNA sites, both EcoRI and EcoRV endonucleases appear to drive the DNA into a rather unfavorable conformation (51). Sequence specific phosphate contacts in the EcoRI-substrate complex anchor and orient protein recognition elements within the major groove of the DNA, and they also act as clamps to stabilize the kinked DNA conformation in the complex. Moreover, the interference with any one of these three phosphates (six per duplex) causes a large effect on binding energy and must reflect the cooperative loss of other contacts (52).

As the structures of DNA-restriction endonuclease complexes have been solved for only a few endonucleases (EcoRI, EcoRV, BamHI, and PvuII) (47) and additional indirect data on the DNA bending and interaction pattern in specific complexes are available only for a limited number of restriction enzymes, the extended screening for the susceptibility of restriction enzymes to THPs inhibition might serve as a simple tool to gain preliminary information on the structure of DNA-protein interface.

The overall standard binding free energy (ΔG°bind) is the net of favorable and unfavorable energetic contributions. In the case of EcoRI endonuclease, these contributions were recently summarized for both the “specific” recognition complex at the GAATTG site and the nonspecific complex (54). Consequently, the net free energy of specific binding includes favorable contributions from protein contacts with DNA bases, phosphate contacts, and release of bound water from nonpolar surfaces and unfavorable contributions from restriction of rotational and translational freedom of the protein and DNA, DNA distortion, protein conformation, etc. (54).

One of the contributions that is favorable for binding energetics results from electrostatic coulombic interactions between positively charged protein side chains and negatively charged DNA phosphates. Coulombic forces are inversely proportional to the dielectric constant of a medium and distances between two interacting charges. THP molecules most likely increase the dielectric constant of the medium like other zwitterionic compounds (25) and, if not repelled from protein or DNA surface upon enzyme-DNA complex formation, may cause a steric interference to the decrease of distances of charges interaction. Both effects will eventually lead to a decrease in favorable energetic contributions of protein-phosphate contacts. The inability of the methylated THP derivatives to inhibit DNA cleavage by EcoRI could be explained by the loss of zwitterionic
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Table I

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Recognition site</th>
<th>DNA substrate</th>
<th>Restriction enzyme</th>
<th>Recognition site</th>
<th>DNA substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td>5’...G↓AATT C....3’</td>
<td>pGEM1 (1 site)</td>
<td>AvuI</td>
<td>5’...C↓CCGG G....3’</td>
<td>pGEM1 (1 site)</td>
</tr>
<tr>
<td></td>
<td>3’...C TTAA ↑G....5’</td>
<td>pBR322 (1 site)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SspI</td>
<td>5’...AAT ↓ATT.....3’</td>
<td>pBR322 (1 site)</td>
<td>EcoRV</td>
<td>5’...G PuGCPy ↑C....5’</td>
<td>Lambda DNA (8 sites)</td>
</tr>
<tr>
<td></td>
<td>3’...TTA ↑TAA.....5’</td>
<td>pGEM1 (1 site)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smal</td>
<td>3’...CCC ↓GGG.....3’</td>
<td>pGEM1 (1 site)</td>
<td>HindIII</td>
<td>3’...CTA ↓TAG.....3’</td>
<td>pBR322 (1 site)</td>
</tr>
<tr>
<td></td>
<td>3’...GGG ↑ CCC.....5’</td>
<td>λ DNA (3 sites)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SgrAI</td>
<td>5’...CG ↓CCGG TG...3’</td>
<td>pBR322 (1 site)</td>
<td>PvuII</td>
<td>5’...CAG ↓CTG.....3’</td>
<td>pBR322 (1 site)</td>
</tr>
<tr>
<td>DraI</td>
<td>3’...GC GGCC ↓AC....5’</td>
<td>pGEM1 (3 sites)</td>
<td>BamHI</td>
<td>3’...GTC ↓GAC.....5’</td>
<td>pBR322 (1 site)</td>
</tr>
<tr>
<td></td>
<td>5’...TTT ↓AAA.....3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3’...AAA ↑TTT.....5’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Figure 8](http://www.jbc.org/)

**Fig. 8. Effect of THP(A) on the cleavage of λ DNA by AvaI restriction endonuclease.** λ DNA (1 µg) was cleaved by 6 units of AvaI restriction endonuclease (minimal amount of enzyme that led to complete cleavage in 1 h), in 10 µl of the reaction mixture. The arrow indicates the position of the band which appear as a result of preferential inhibition of λ DNA cleavage by AvaI at its CTCGAG recognition site.

The water interface would also decrease its favorable energetic contribution. This interference might be crucial for the specific complexes, in which additional energy is necessary for the DNA distortion by kinking, bending, and unwinding, and interactions with specific phosphate groups play an additional role in stabilizing a kinked DNA conformation in the protein-DNA complex. This interference depends on whether THPs belong to the groups of preferentially excluded or preferentially interacting solutes (56); in other words, on the affinity of THPs to the protein surface in comparison to that of water. For example, so-called, solvophobic compounds (one of the categories of the preferentially excluded) make contact of the nonpolar regions of the protein with the solvent more unfavorable thermodynamically than their contact with water, and thus render the hydrophobic effect even stronger (56).

It was noted that most of the natural osmolytes belong to the class of compounds that are protein stabilizers and are preferentially excluded by a nonspecific mechanism, such as the increase in surface tension or the solvophobic effect (56). Still, it is worth noting that the delocalized positive charge of THPs is not characteristic of other zwitterionic osmolytes, such as proline and betaine, nor is the ability to interfere with protein-DNA interaction. In order to further refine the mechanism of THPs interference with specific DNA-protein interaction, data on the dielectric constant of THPs solutions and THPs localization in the solution relatively to the macromolecules surface (preferential exclusion or preferential interaction) has to be collected.

**In Vivo Relevance of the Observed THP Effect—**Inhibition of endonuclease-catalyzed DNA cleavage by DNA-binding ligands has been described previously for a number of drugs, *e.g.* proflavin, olivomycin, ethidium bromide, actinomycin D, and distamycin A (26). Yet these inhibitors do not normally occur in microbial cells at physiological conditions. Even in microorganisms producing antibiotics, these metabolites are actively excreted from the cell to avoid suicide (57). On the contrary, polyamines (putrescine, spermine, and spermidine), prokaryotic histone-like proteins NS1 and NS2 (27), and polyphosphate (28) are common bacterial cell constituents that inhibit restriction endonuclease-catalyzed DNA cleavage. Now we can add THPs to this list of substances. However, THPs exhibit a number of distinct features compared with those of polyamines: (i) DNA cleavage by restriction endonucleases is not activated by low concentrations of THPs as it is by spermine and spermidine; (ii) although polyamines bind to double-stranded DNA
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and stabilize it, binding of THPs to DNA was not detected, and THP(B) significantly destabilized DNA; (iii) THPs do not cause any apparent condensation or precipitation of DNA as do polyamines at concentrations of 0.2–0.3 mM (27). The intracellular concentration of THP(A), as shown for a number of Streptomyces strains, ranges from 15–20 mM at normal growth conditions to 50–150 mM as a response to osmotic stress or to 38–44 mM at elevated temperatures (7). The finding that THP(A) inhibits SgrAI endonuclease in the range of 2–4 mM, similarly to other tested endonucleases, suggests that the restriction enzymes of the Streptomyces bacteria are not resistant to the inhibition by THP(A). Such a coexistence of restriction enzyme and its inhibitor in concentrations sufficient to completely suppress DNA cleavage in vitro raised the question of how the functions of restriction system are retained. We have shown that spermine (and most likely other polyamines) can compensate the inhibition of EcoRI binding and cleavage of DNA, by moderate concentrations of THP(A). Yet THP(A) concentrations higher than 10 mM were compensated only partially by spermine, suggesting that THPs still can lead to alleviation of restriction in vivo. Indeed, heat sensitivity of the restriction system in vivo has been reported for a number of Streptomyces strains (58, 59) and for Corynebacterium glutamicum (60). It is tempting to propose that the alleviation of restriction in these cases may be the result of induction of the intracellular synthesis of THPs under heat stress. It was suggested by Schafer et al. (60) that under stress, restriction would be alleviated, and the cell could acquire foreign genetic information more easily, possibly enhancing the capability to deal with the particular environmental requirement. In addition, high endonuclease activity is lethal for bacteria, if it is not balanced by the activity of corresponding methylase and DNA ligase (21); therefore, the restriction alleviation might also be a part of bacterial survival strategy under stress. Finally, the potential ability of THPs to alleviate restriction and to be efficiently taken up by bacteria (7, 9) may be of use in genetic manipulation with microorganisms. However, the correlation of the effect of THPs on restriction endonuclease activity in vitro, and the modulation of the restriction system in vivo needs to be further investigated.

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REFERENCES

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Effect of Tetrahydropyrimidine Derivatives on Protein-Nucleic Acids Interaction: TYPE II RESTRICTION ENDONUCLEASES AS A MODEL SYSTEM
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