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An interaction between gramicidin and the σ subunit of RNA polymerase

(trypsin cleavage/sporulation/peptide antibiotics/RNA nucleotidyltransferase)

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ABSTRACT **Gramicidin, a peptide antibiotic produced by *Bacillus brevis*, inhibits initiation of transcription by RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6). We show here that the presence of gramicidin causes an increase in the rate of cleavage of the σ subunit of *Escherichia coli* RNA polymerase by trypsin, although it does not alter the cleavage rate of any of the core subunits. Furthermore, whereas isolated σ is cleaved much faster than is σ in holoenzyme, gramicidin substantially decreases the trypsin cleavage rate of isolated σ. Inhibition of RNA polymerase activity by gramicidin is consistent with a σ-specific effect: the antibiotic is a strong inhibitor of transcription of T7 phage DNA, which requires σ for activity, but it has little effect on transcription of σ-independent templates, such as poly(dA-dT)·poly(dA-dT) and calf thymus DNA. These results are discussed in light of the hypothesized role for gramicidin in the initiation of sporulation of *B. brevis*.**

Gramicidin is a linear pentadecapeptide produced by certain cultures of *Bacillus brevis* during the transition from vegetative growth to sporulation. It is one of a class of peptide antibiotics whose antibacterial properties may not represent their natural function in the synthesizing organism (1, 2). Although the antibacterial activity of gramicidin is attributed to its ability to increase the permeability of membranes to monovalent cations (3), Sarkar and Paulus have proposed that gramicidin promotes the shift from vegetative growth to sporulation (1). This conclusion is supported by several lines of evidence: (*i*) Gramicidin is synthesized prior to the onset of sporulation at the end of exponential growth (1). (*ii*) Mutants of *B. brevis* that are unable to synthesize gramicidin are unable to form normal spores unless provided with the antibiotic at the end of exponential growth (4). (*iii*) Commercial preparations of gramicidin inhibit the *in vitro* activities of RNA polymerases (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) from a wide variety of organisms (1, 2, 5, 6).

Mukherjee and Paulus have speculated that gramicidin functions by turning off the transcription of vegetative genes that are not essential for sporulation (4). Tyrocidin, another small peptide antibiotic produced by *B. brevis*, has been shown to inhibit transcription by forming a complex with the DNA template (7, 8), but attempts to demonstrate a similar interaction between gramicidin and DNA have been unsuccessful (2). Similarly, attempts to demonstrate complex formation between gramicidin and *B. brevis* RNA polymerase have also failed (2). Here we show that one or more components of commercial gramicidin preparations do in fact interact with *Escherichia coli* RNA polymerase, specifically with the σ subunit.

σ is directly involved in promoter recognition and directs transcriptional specificity (9–11). In *B. subtilis*, the transcriptional shift from vegetative to sporulation-specific genes is accomplished by the removal of vegetative σ subunit and its replacement with a new polypeptide that confers an altered transcriptional specificity on the enzyme (12). On the basis of the findings reported here with *E. coli* RNA polymerase, we hypothesize that gramicidin could be responsible for displacement of the vegetative σ subunit.

MATERIALS AND METHODS

Materials. Sodium dodecyl sulfate ($NaDodSO_4$) was from British Drug House (Poole, England). Sodium tetradecyl sulfate ($NaTetSO_4$) was from Pfaltz and Bauer (Stamford, CT). Trypsin (bovine pancreas, A grade) was from Calbiochem; trypsin inhibitor (soybean) was from Boehringer Mannheim. Nucleoside triphosphates and poly(dA-dT)·poly(dA-dT) were purchased from P-L Biochemicals. [^{3}H]UTP (25 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was from ICN Pharmaceuticals. Sephadex G-50 fine was purchased from Pharmacia. Calf thymus and salmon sperm DNAs were from Sigma. Acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Bio-Rad.

RNA polymerase core and holoenzyme were purified from *E. coli* MRE 600 as described (13). The σ subunit was purified from holoenzyme by the method of Lowe *et al.* (14). T7 phage DNA was purified by the method of Thomas and Abelson (15).

All of the experiments reported here were performed with a commercial preparation of gramicidin D, purchased from Boehringer Mannheim. No attempt either to fractionate or to analyze this preparation has been made. Hence we do not know what the components that have the activities described below are. We use the term "gramicidin" throughout to refer to the active molecule(s).

Trypsin Cleavage. RNA polymerase was cleaved in standard buffer (10 mM Tris·HCl, pH 8.0/0.1 mM EDTA/0.1 mM dithioerythritol/0.15 M KCl) at the concentrations indicated in the individual figure legends. At the indicated times, 10-μl aliquots were removed to tubes containing 5 μl of 24 μM trypsin inhibitor. The samples were mixed with 10 μl gel sample buffer containing 2% $NaDodSO_4$ and subjected to denaturing gel electrophoresis according to the procedure of Laemmli (16). Gels were stained with Coomassie brilliant blue, destained by diffusion, and scanned by using a Helena Quick Scan microdensitometer, which automatically integrated the areas under each peak.

Column Centrifugation. The binding of gramicidin to RNA polymerase was measured by using the column centrifugation technique described by Penefsky (17). Recovery of proteins in

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Abbreviation: $NaTetSO_4$, sodium tetradecyl sulfate.

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the effluent was always determined by the modified Lowry assay described by Peterson (18).

Assay of RNA Polymerase Activity. The assay mixture (in 100 μl) was 15 mM Tris·HCl (pH 8.0)/7.5 mM $MgCl_2$/5 mM 2-mercaptoethanol/30 mM KCl/1.2 mM ATP, CTP, and GTP/0.4 mM [^{3}H]UTP (380 mCi/mmol), and DNA as indicated in the individual figure legends. After incubation for 10 min at 37°C, RNA synthesis was terminated by precipitation with 3 ml of 5% trichloroacetic acid. Precipitates were collected on nitrocellulose (Millipore type HA) filters and assayed for radioactivity in Omnifluor (New England Nuclear)/toluene scintillation fluid.

RESULTS

Effect of Gramicidin on Trypsin Cleavage of *E. coli* RNA Polymerase. In order to identify a subunit-specific effect of gramicidin, we measured the rates at which each of the subunits in holoenzyme, $\alpha_2\beta\beta'\sigma$, was cleaved by trypsin in the absence and presence of the antibiotic. When the samples were subjected to electrophoresis on $NaTetSO_4$ gels as described (13), no effects of gramicidin on trypsin cleavage of any of the core subunits (α, β, β') were observed (data not shown). However, we found that the σ subunit disappeared completely when holoenzyme was analyzed by electrophoresis on $NaTetSO_4$ gels in the presence of gramicidin. When purified σ was subjected to electrophoresis on $NaTetSO_4$ gels in the presence of gramicidin, again the σ subunit failed to appear. The total lack of resolution of σ on $NaTetSO_4$ gels in the presence of gramicidin may be a phenomenon dependent on a specific gramicidin–σ–$NaTetSO_4$ gel system interaction. When samples of holoenzyme (or purified σ subunit) were cleaved by trypsin in the presence of gramicidin and subjected to electrophoresis on $NaDodSO_4$ gels, the σ subunit and its major trypsin cleavage product appeared normally. The data in Fig. 1 show that the presence of gramicidin results in an increase in the cleavage rate of σ. Thus both the gramicidin-induced loss of σ from $NaTetSO_4$ gels and the alteration in the rate of trypsin cleavage of σ by the antibiotic suggest a specific interaction between gramicidin and σ.

Such an interaction is further supported by the following experiment. The σ subunit was removed from holoenzyme and cleaved with trypsin in the absence and presence of gramicidin. We found that whereas free σ is cleaved much more rapidly than holoenzyme σ, the presence of gramicidin substantially slows this accelerated rate (Fig. 2). Hence the antibiotic destabilizes σ in holoenzyme but stabilizes free σ against trypsin cleavage. This raises the possibility that gramicidin induces a single intermediate cleavage rate that is independent of the presence of the core subunits. However, several trypsin cleavage experiments with holoenzyme and purified σ (unpublished observations) have convinced us that the core subunits do slow the cleavage of σ in the presence of gramicidin. The data fit the following cleavage rate scheme: free σ > free σ + gramicidin > holoenzyme σ + gramicidin > holoenzyme σ. The concentration of gramicidin required to change the cleavage rate of σ by 50% with both holoenzyme and free σ was found to be approximately 100 μM.

Effect of Gramicidin on Transcription. Because our evidence showed a physical interaction between gramicidin and σ, it seemed likely that transcriptional activity would be inhibited only when transcription was dependent on σ. Therefore the effect of increasing concentrations of gramicidin on both core and holoenzyme was measured with a template that requires σ for maximal activity, T7 DNA (19). We also measured the effect of increasing concentrations of gramicidin on transcriptional activity with two σ-independent templates, poly(dA-dT)·poly(dA-dT) and calf thymus DNA (20, 21). The results are shown in Fig. 3. With the templates that do not require σ for activity gramicidin had very little inhibitory effect (Fig. 3 *A* and *B*). However, on the σ-dependent template, T7 DNA, gramicidin strongly inhibited activity of holoenzyme (Fig. 3*C*). On this template a small amount of core enzyme activity is also lost, perhaps due to a trace amount of contaminating σ in the core preparation. The data show that gramicidin is a powerful inhibitor of RNA polymerase, but only under conditions in which σ is required. Fifty percent inhibition occurs at approximately 10 μM.

Holoenzyme-Gramicidin Interaction Is Sufficiently Stable to Withstand Column Centrifugation. In this section we present evidence that a stable interaction exists between gramicidin and the holoenzyme. We mixed gramicidin with holoenzyme and subjected the mixture to centrifugation in a Sephadex G-50 fine column as described by Penefsky (17). If there is no interaction between RNA polymerase and gramicidin, then the enzyme will be rapidly sedimented through the column, whereas the much smaller gramicidin will be retained by the

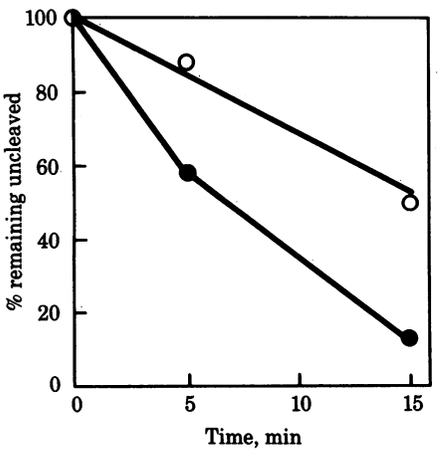

FIG. 1. Effect of gramicidin on the time course of trypsin cleavage of the σ subunit of holoenzyme. Holoenzyme (0.56 μM) in 50 μl of standard buffer plus 27% (vol/vol) glycerol was cleaved at room temperature (21°C) with trypsin at a final concentration of 1.2 μM in the presence (●) and absence (○) of 130 μM gramicidin. Samples were then treated as described in the text. Values are presented as percent of initial (i.e., no trypsin added) subunit remaining uncleaved.

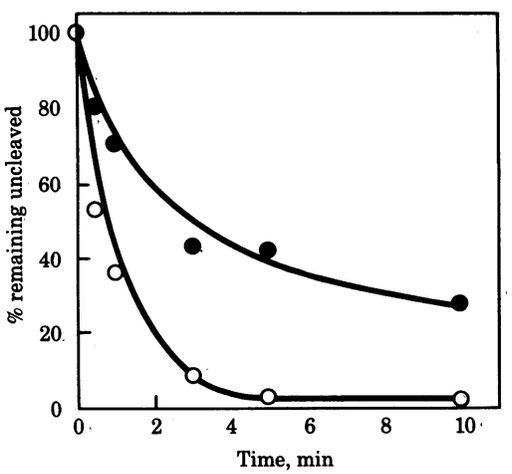

FIG. 2. Effect of gramicidin on trypsin cleavage of purified σ subunit. Isolated σ subunit (1.8 μM) in 50 μl of standard buffer plus 18% (vol/vol) glycerol was cleaved at 0°C with trypsin at a final concentration of 0.8 μM in the presence (●) and absence (○) of 108 μM gramicidin.

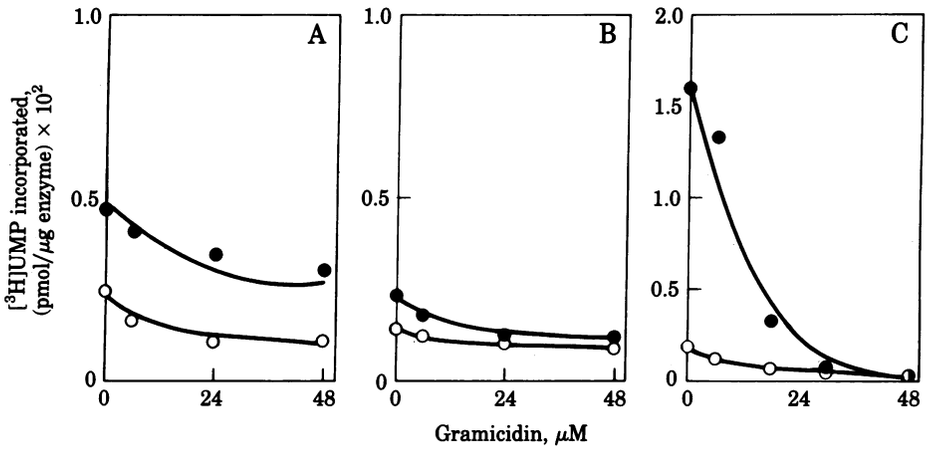

FIG. 3. Effect of gramicidin on transcription of core and holoenzyme. Core (○) (1.2 μg) and holoenzyme (●) (0.27 μg) were assayed for activity on 2.5 μg of poly(dA-dT)·poly(dA-dT) (*A*), 3 μg of calf thymus DNA (*B*), or 0.4 μg of phage T7 DNA (*C*) in the presence of increasing amounts of gramicidin. Gramicidin was added from a stock solution dissolved in ethanol; equivalent amounts of ethanol were present in all incubation mixtures.

gel matrix. If there is a relatively stable interaction between the antibiotic and RNA polymerase, then the enzyme and gramicidin will appear in the excluded volume in the column effluent. Column centrifugation effluents were assayed for the presence of gramicidin by determining transcription activity on both σ-dependent and σ-independent templates.

Table 1 shows that, in the absence of gramicidin, holoenzyme is >4-fold more active on the σ-dependent template. Conversely, in the presence of gramicidin, the enzyme is >10-fold more active on the σ-independent template. Because all of the RNA polymerase subunits, including σ, were present in the column effluents (data not shown), this indicates that gramicidin has passed through the column along with the holoenzyme. It inhibits transcription of σ-dependent templates, while still permitting expression of residual core activity on the σ-independent template. Gramicidin does not appear in the excluded volume in the absence of holoenzyme (unpublished observations).

Do Gramicidin and DNA Compete for the Same Site on RNA Polymerase? It has previously been suggested that gramicidin competes with DNA for the same site on RNA polymerase (5). To determine whether the inhibition was indeed competitive, transcription was performed with increasing concentrations of T7 phage DNA after preincubation of holoenzyme with gramicidin. Fig. 4 is a double-reciprocal plot of RNA synthesis as a function of DNA concentration in the presence and absence of gramicidin. Although the antibiotic decreases the amount of RNA synthesis, the half-maximal titration of template binding sites is unaffected. Thus, gramicidin and DNA appear to interact with RNA polymerase independently.

More evidence for this conclusion is provided by the following trypsin cleavage experiment. Whereas gramicidin speeds the cleavage of σ in holoenzyme, DNA slows its cleavage (22). Fig. 5 shows the results of a trypsin cleavage experiment in which the order of addition to RNA polymerase of gramicidin and DNA was varied. When gramicidin alone was added, the cleavage of σ was rapid; when poly(dA-dT)·poly(dA-dT) alone was added, the cleavage of σ was slow. When both were added, regardless of which was added first, the σ cleavage was slow. Thus even when gramicidin is added first, at a concentration that completely inhibits transcription activity and that saturates the trypsin cleavage experiment, polymerase is still able to interact with the DNA.

Table 1. Holoenzyme–gramicidin complex withstands column centrifugation

Incubation	Sample	Polymerase activity, pmol [³H]UMP incorporated/ pmol enzyme: Salmon sperm DNA	T7 DNA	Salmon sperm/T7
Without gramicidin	Before	8.94	66.14	0.14
	Effluent	6.68	27.29	0.24
With gramicidin	Before	2.04	0.20	10.2
	Effluent	0.92	0.08	11.5

Holoenzyme (0.6 μM) in 100 μl of standard buffer plus 27% (vol/vol) glycerol was preincubated 10 min at 37°C in the presence or absence of 110 μM gramicidin. Aliquots (70 μl) were then applied to centrifuge columns. The effluent and unloaded portion (before) were assayed for protein recovery and transcriptional activity on salmon sperm DNA (97 μg/ml) and phage T7 DNA (23 μg/ml).

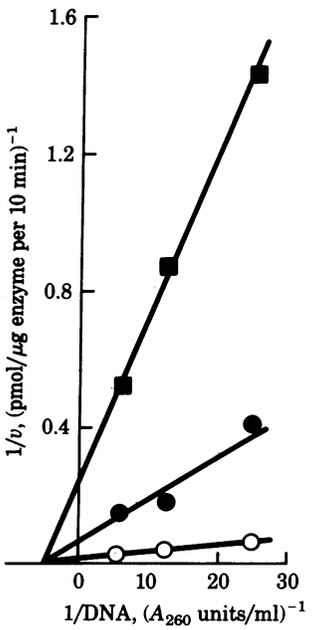

FIG. 4. Effect of DNA concentration on the rate of RNA synthesis in the absence and presence of gramicidin. RNA polymerase holoenzyme (0.27 μg) was assayed at the phage T7 DNA concentrations indicated in the absence (○) or presence of gramicidin at 6.5 μM (●) or 9.75 μM (■).

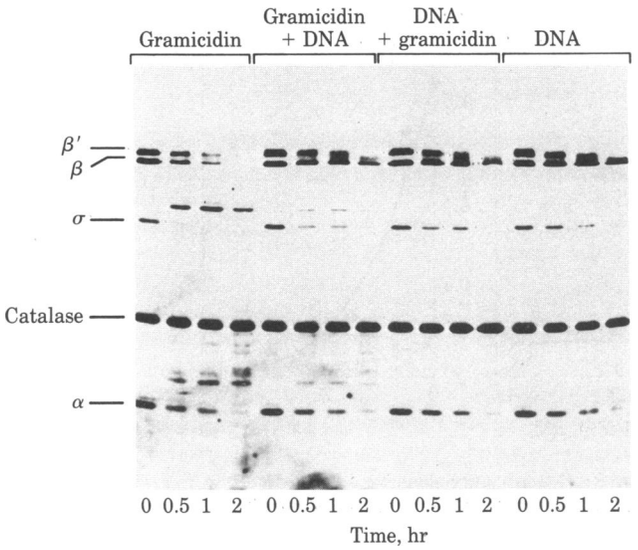

FIG. 5. Effect of gramicidin and poly(dA-dT)·poly(dA-dT) on the time course of trypsin cleavage of RNA polymerase. Holoenzyme (0.59 μM) in 40 μl of standard buffer plus 27% (vol/vol) glycerol was preincubated 5 min at 37°C after addition of gramicidin (130 μM), poly(dA-dT)·poly(dA-dT) (47 μg/ml), or both. Samples were cleaved at room temperature with trypsin at a final concentration of 2 μM.

DISCUSSION

Interaction of Gramicidin with σ. The trypsin cleavage experiments described here demonstrate that a component of a commercial gramicidin preparation interacts with both free σ and holoenzyme. This component, which we call "gramicidin," had different effects on the σ subunit, depending on whether or not σ was bound to core polymerase: it decreased the rapid cleavage rate of free σ, but increased the slow cleavage rate of the σ subunit in holoenzyme. These results argue for a direct interaction between gramicidin and σ.

Our measurements of inhibition of transcriptional activity by gramicidin are consistent with this conclusion. Only transcriptional activity dependent on σ is strongly inhibited by the antibiotic. The weakness of the experiments reported here is that we have not identified the active component(s) of the gramicidin preparation. The fact that the levels of gramicidin required for inhibition of activity (50% inhibition at 10 μM) and for trypsin cleavage (50% reduction in $t_{1/2}$ at 100 μM) are quite different suggests the possibility that one component is responsible for the inhibition of RNA polymerase activity, while another may be responsible for the alterations in the rates of trypsin cleavage. However, the fact that both turned out to be σ-specific argues for at least a close relationship between the active components.

Function of Gramicidin During Development. Our experiments were performed with RNA polymerase from *E. coli*, which is not the natural target of gramicidin. The extrapolation of conclusions based on experiments with the *E. coli* enzyme to explain the biology of sporulation in *B. subtilis* depends on the assumption that the σ subunits from the two enzymes are homologous. The validity of this assumption is supported by the observation that σ isolated from either species can complement either core enzyme for *in vitro* transcription of a variety of natural templates (23). Furthermore, the RNA polymerases isolated from *B. brevis* and *E. coli* have been shown to be equally sensitive to gramicidin (6). Thus it is worthwhile to extrapolate our findings to discuss the possible action of gramicidin during sporulation of *B. brevis*.

Mukherjee and Paulus (4) have proposed that gramicidin plays an obligate role in the transition from vegetative growth to sporulation in *B. brevis*. Further, Tjian et al. (24) have concluded on the basis of experiments with antibody to σ factor that sporulating *B. subtilis* contains a component that interferes with σ. Our finding that gramicidin interacts with σ subunit from *E. coli* suggests the possibility that gramicidin could be related to the inhibitor detected by Tjian et al. (24). Because we found a specific interaction between gramicidin and σ, it seems reasonable that gramicidin could act directly to modify the affinity of σ for the core enzyme. This modification might permit other transcription specificity factors, such as σ^{37} identified in *B. subtilis*, to interact with the polymerase and stimulate selective transcription of genes responsible for the transition from vegetative growth to sporulation (12, 25).

Wiggs et al. (26) have recently demonstrated the presence of another vegetative σ factor in *B. subtilis*, which confers a new transcriptional specificity on *B. subtilis* core enzyme. It would be interesting to determine whether this enzyme can be inhibited by gramicidin.

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