Streptococcus mutans Dextranucrase: Effect of Cerulenin on Lipid Synthesis and Enzyme Production

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The effect of the fatty acid synthesis inhibitor cerulenin on growth and dextranucrase (EC 2.4.1.5) production by *Streptococcus mutans* 6715 was analyzed. Growth was markedly inhibited by less than 1 μ g of the antibiotic per ml. Under conditions where cerulenin did not inhibit amino acid incorporation into protein but did block acetate metabolism into lipid, the production of extracellular dextranucrase was suppressed. Inhibition was not due to a direct effect of the antibiotic on the enzyme or the lack of release of enzyme from the bacterial cell surface. Gel column chromatography demonstrated that enzyme produced in the presence of cerulenin was highly aggregated, similar to the control enzyme. Although the addition of lysophosphatidylcholine to enzyme which had been synthesized in the presence of cerulenin stimulated glucan formation from sucrose, the increase was not greater than that produced with the control enzyme. The differential inhibition of dextranucrase production by cerulenin indicates that enzyme secretion requires the production of lipid and may reflect the mechanism by which this enzyme is transported from the bacterial cell.

Streptococcus mutans dextranucrase activity appears to be directly related to the cariogenic potential of this bacterium (21). Recent investigations in this laboratory have demonstrated that the enzyme can interact with phospholipids with a concomitant elevation in the rate of glucan production from sucrose (12, 19, 21). Additional studies have indicated that the availability of fatty acids may influence dextranucrase production by *S. mutans* (16) and that surface active agents such as Tween 80 can stimulate enzyme production and activity (22, 23). These observations indicate that dextranucrase may be synthesized and excreted by *S. mutans* via mechanisms similar to those utilized by *Bacillus licheniformis* for the enzyme penicillinase (6, 15, 24, 25) and *Bacillus subtilis* for the enzyme levansucrase (3). In both of these cases, lipid synthesis has been shown to be required for extracellular enzyme production (3, 7).

The antibiotic cerulenin is an inhibitor of fatty acid synthesis in bacteria (1, 5, 17) and has recently been used to study the role of lipid production in macromolecular synthesis and cell division in *Streptococcus faecalis* (2). Preliminary studies on the effect of cerulenin on *S. mutans* have been presented (S. Leung, S. Harlander, and C. Schachtele, J. Dent. Res. 58A:309, 1979; C. Wittenberger and A. Wolf, J. Dent. Res. 58A:337, 1979). In this communication we present studies in which this inhibitor has been used to analyze the role of lipid synthesis in

extracellular dextranucrase production by *S. mutans* 6715.

MATERIALS AND METHODS

Bacterium and growth conditions. The particular *S. mutans* strain used in these studies (6715) has been described previously (12). It was originally obtained from the Forsyth Dental Center, Boston, Mass., and its cariogenicity was checked in the gnotobiotic rat model system by S. Michalek (University of Alabama at Birmingham). Growth of the bacterium was anaerobic at 37°C in buffered and yeast extract-supplemented Trypticase soy broth (TSB) containing either glucose (8) or fructose (9). The latter carbohydrate was used to obtain cultures producing elevated levels of dextranucrase (9). During the time course studies, cultures were manipulated in a 37°C room, with anaerobic conditions being maintained by flushing rubber-capped tubes or flasks with a stream of gas (N₂, 95%; CO₂, 5%). Turbidity was monitored with a Klett-Summerson colorimeter (no. 66 filter) with broth as a blank. Viable bacteria were quantitated by briefly sonicating samples of cultures, diluting in broth medium without added carbohydrate, and plating onto TSB supplemented with glucose (0.25%) and agar (1.5%). Incubation of plates was for 48 h at 37°C in an atmosphere of 80% N₂, 10% CO₂, and 10% H₂.

Cerulenin. The antibiotic (Sigma Chemical Co., St. Louis, Mo.) was stored in dry form at -20°C. Before each set of experiments, cerulenin was dissolved in absolute ethanol and maintained in the cold. In all of the growth and enzyme studies, controls were always run with the addition of absolute ethanol containing no antibiotic. The addition of ethanol to a

maximum concentration of 2% had no detectable effect on cell growth as measured by culture turbidity.

Dextranucrase assay. Crude cell-free supernatants were prepared by centrifuging aliquots of cultures at $10,000 \times g$ for 15 min at 4°C . Samples were stored at 4°C until utilized for enzyme assay. Ammonium sulfate-precipitated and dialyzed enzyme preparations were prepared as described previously (8).

The basic assay system for quantitating total alcohol-insoluble glucan production from [^{14}C]sucrose has been described in detail (11). When additional components were tested in the assay, the conditions utilized in a previous study were employed (12). Activity was monitored by mixing 25 μl of diluted or undiluted enzyme and 25 μl of test compound, organic solvent, or buffer (0.01 M sodium acetate, pH 5.5) before initiating the assay by addition of 75 μl of sucrose-containing reaction mixture made up with T10 dextran at a concentration of 20 μM . During incubation at 37°C , four 25- μl aliquots were removed at various times, placed onto paper disks, and processed for quantitation of radioactive glucan as described previously (11). In crude cell-free culture supernatants, the counts incorporated at the 60-min time point were normally used to express the activity present in the sample.

The *S. mutans* cell-associated dextranucrase activity was assayed by using washed cells as described previously (18).

Protein and lipid synthesis. To monitor protein and lipid synthesis, rapidly growing cells at a Klett reading of 40 to 45 were supplied with either $U\text{-}^{14}\text{C}$ -labeled L-amino acids (NEC-445; New England Nuclear Corp., Boston, Mass.; final concentration, 0.5 $\mu\text{Ci/ml}$) or [$1,2\text{-}^{14}\text{C}$]acetate (56 mCi/mmol; New England Nuclear; final concentration, 0.5 $\mu\text{Ci/ml}$) and placed into a series of flasks containing cerulenin or absolute ethanol. During incubation at 37°C , aliquots of the culture were obtained to determine incorporation of radioactivity. For protein synthesis 3-ml samples were mixed into 3 ml of iced 10% trichloroacetic acid. After storage for at least 90 min, duplicate 1.0-ml aliquots of each sample were filtered through 0.45- μm filters (HAWP type, Millipore Corp., Bedford, Mass.), and the filters were washed three times with 15 ml of cold 5% trichloroacetic acid. The filters were air dried and assayed for radioactivity as described previously (11). For acetate incorporation 3-ml aliquots were similarly placed into an equal volume of iced 10% trichloroacetic acid. After storage the trichloroacetic acid-precipitable material was sedimented by centrifugation, suspended in 6 ml of iced 5% trichloroacetic acid, and collected again by centrifugation. One additional wash was performed, and the resulting pellet was incubated at 45°C for 30 min after suspension in 2 ml of ethanol-ether (1:1, vol/vol). Insoluble material was again pelleted and washed with 1 ml of ethanol-ether. The supernatants were combined and stored at 4°C . Radioactivity in each sample was determined by placing 0.1-ml aliquots onto Whatman 3MM filter paper disks, drying the disks, and analyzing them in a scintillation counter as described previously (11).

Gel filtration chromatography. Fractionation of ammonium sulfate-precipitated and dialyzed enzyme preparations on Bio-Gel A 1.5 M was performed as described previously (9, 10, 20).

RESULTS

Effect of cerulenin on growth and cell viability. Inoculation of *S. mutans* into fructose broth cultures containing the antibiotic demonstrated that cell growth was inhibited by concentrations of cerulenin as low as 0.1 μg per ml (Fig. 1). Complete inhibition of growth was obtained at concentrations greater than 1.0 μg per ml. When the same experiment was repeated with cells utilizing glucose rather than fructose as a carbon source, it was demonstrated that the bacteria were slightly more sensitive with complete inhibition of growth occurring at a cerulenin concentration of 0.5 μg per ml (data not shown).

When rapidly growing cells were transferred into fructose-broth cultures containing cerulenin, the antibiotic only slightly suppressed the increase in turbidity at a concentration of 5 μg per ml (Fig. 2A). At a concentration of 10 μg per ml, the antibiotic decreased the final level of growth by ca. 25%, and at 20 μg per ml the increase in turbidity was more rapidly effected with the final readings representing 50% of the control value.

The inhibition of cell growth was not due to a bactericidal effect on *S. mutans* since rapidly growing cells treated with up to 20 μg of cerulenin per ml for 90 min in glucose broth were still viable when plated onto nutrient agar plates. No increase in viable cell number was observed dur-

FIG. 1. Effect of cerulenin on the growth of *S. mutans* 6715. An 18-h fructose broth culture was diluted 1:50 into tubes containing the same medium and increasing concentrations of the antibiotic. Turbidity was measured after 24 h of incubation at 37°C . Growth in the absolute ethanol control was 310 Klett units.

FIG. 2. Effect of cerulenin on growth (A) and dextranucrase production (B) by *S. mutans*. Fructose broth cultures were inoculated to a Klett reading of 45 in the absence (○) and presence of cerulenin at concentrations of 5 (●), 10 (Δ), and 20 (■) μg of cerulenin per ml. During anaerobic incubation at 37°C, samples of the cultures were removed and assayed for growth and soluble dextranucrase activity.

ing the 90-min interval, while a control culture showed a 2.5-fold increase in viable bacteria (data not shown). Removal of cerulenin by filtration and cell washing allowed the bacteria to rapidly grow when placed in fresh medium.

Effect of cerulenin on protein and lipid synthesis. Under conditions where growth in glucose broth of *S. mutans* was partially inhibited by the presence of cerulenin (Fig. 3), it could be demonstrated that the antibiotic had little effect on the differential rate of protein synthesis as measured by the incorporation of ¹⁴C-labeled amino acids into trichloroacetic acid-insoluble material (Fig. 4). Under conditions in which growth was significantly inhibited by 10 μg of cerulenin per ml, the production of protein was directly related to increasing cell mass as measured turbidometrically. In contrast, under identical conditions, the formation of lipid as quantitated by the incorporation of [¹⁴C]acetate (Fig. 5) is markedly inhibited by the presence of cerulenin at concentrations of 1.0 and 10 μg per ml. The differential rate of lipid formation was inhibited ca. 85% by 10 μg of cerulenin per ml under conditions where protein production was only slightly affected.

Analysis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) production with [³H]-thymidine and [³H]uridine in the presence and absence of cerulenin under conditions similar to those presented in Fig. 3 demonstrated that nucleic acid synthesis in *S. mutans* was not inhibited by 20 μg of the antibiotic per ml for at least 60 min (data not shown).

Dextranucrase production in the presence of cerulenin. The results presented in Fig. 2B allow a direct comparison of the effect of cerulenin on extracellular dextranucrase production and cell growth in fructose broth (Fig. 2A). Although the antibiotic at 5 μg per ml has a minimal effect on the increase in *S. mutans* cell mass, there is a clear inhibition of the quantity of dextranucrase released into the culture fluid. The most striking inhibition is in the presence of 20 μg of cerulenin per ml. Enzyme production could not be detected for 60 min after exposure to the antibiotic under conditions in which culture turbidity was increasing at nearly the control rate. After this initial inhibition enzyme activity was found to increase at a low rate under conditions in which there was only a slight increase in cell mass.

Similarly, when the cells grown in glucose broth (Fig. 3) were analyzed for the production of extracellular dextranucrase, a marked inhibition of the differential rate of enzyme formation was observed with both 1.0 and 10 μg of cerulenin per ml (Fig. 6). It is clear that cerulenin has a dramatic effect on dextranucrase secretion into the culture medium under conditions in which lipid synthesis appears to be selectively suppressed relative to cell mass increases and protein synthesis.

Cell-associated dextranucrase activity was similar in cell preparations from *S. mutans* grown in the presence and absence of cerulenin. In each case the level of enzyme was directly

FIG. 3. Effect of cerulenin on growth of *S. mutans* in glucose-containing broth in the absence (○) of cerulenin and in the presence of the antibiotic at concentrations of 1 (●) and 10 (Δ) μg per ml.

FIG. 4. Effect of cerulenin on the differential rate of protein synthesis by *S. mutans*. Samples from cultures identical to those in Fig. 3 except for the presence of radioactive amino acids were removed, and the amount of radioactive protein was determined. Symbols: (○) control; (●) cerulenin at 1 µg per ml; (△) cerulenin at 10 µg per ml.

related to the number of bacteria present as determined by culture turbidity (data not shown). No elevated level of cell-associated enzyme activity was found in cell preparations incubated with cerulenin under conditions which markedly suppressed extracellular enzyme production (Fig. 2).

Cerulenin and dextranucrase activity. Table 1 presents a study demonstrating that the addition of cerulenin at concentrations of up to 20 µg/ml to a crude *S. mutans* dextranucrase preparation had little effect on either the rate or extent of glucan production from sucrose by the enzyme. Cerulenin at similar concentrations was also shown to have no effect on the production of water-soluble and water-insoluble glucans by partially purified forms of the dextranucrase, which produced these types of products (9). It would appear to be unlikely that the inhibitory effect of cerulenin on dextranucrase production (Fig. 2B and 6) was due to a direct effect of the antibiotic on the enzyme.

Enzyme produced in the presence of antibiotic. Table 2 presents a study which demonstrates that the decreased quantities of dextranucrase detected in samples from cultures incubated with cerulenin was not due to a lack of excretion of lipid molecules with the potential to stimulate enzyme activity. Addition of lyso-phosphatidylcholine to the enzyme preparations increased glucan formation, but the elevated activity was correspondingly less than that ob-

served with the control samples from untreated cultures.

When enzyme was prepared from the culture supernatant of cells grown in glucose broth in the presence and absence of cerulenin and

FIG. 5. Effect of cerulenin on the differential rate of lipid synthesis by *S. mutans*. Samples from cultures identical to those in Fig. 3 except for the presence of [^{14}C]acetate were removed and fractionated to determine the quantity of radioactive lipid present. Symbols: (○) control; (●) cerulenin at 1 µg per ml; (△) cerulenin present at 10 µg per ml.

FIG. 6. Differential rate of soluble dextranucrase production by *S. mutans* in the absence (○) and presence of cerulenin at concentrations of 1.0 (●) and 10 (△) µg per ml. Cultures were prepared as described in Fig. 3 with samples removed and tested for soluble dextranucrase activity after removal of the bacterial cells.

TABLE 1. *Effect of cerulenin on crude S. mutans dextranucrase activity*

Assay conditions ^a	Glucan formed (cpm/25 μ l of enzyme) ^b at h:			
	1	2	3	4
Control	3,220	6,070	8,040	11,040
Plus ethanol	2,870	5,460	6,670	9,460
Plus ethanol and cerulenin (10 μ g/ml)	2,920	4,760	6,540	9,770
Plus ethanol and cerulenin (20 μ g/ml)	2,670	5,120	6,620	10,030

^a Absolute ethanol was added to a final concentration of 20%.

^b A cell-free fructose-broth culture supernatant was used as the source of enzyme.

TABLE 2. *Effect of lysophosphatidylcholine (LPC) on S. mutans dextranucrase produced in the absence and presence of cerulenin*

Assay conditions ^a	Glucan formed (cpm/25 μ l of enzyme) ^b	
	Minus cerulenin ^c	Plus cerulenin ^c
Control	6,750	5,750
Plus LPC	11,550	9,000

^a Assays were for 180 min at 37°C in the presence of dextran T10. When present, LPC was added to a final concentration of 1 mM.

^b Cell-free fructose-broth culture supernatants were used as a source of enzyme. Cerulenin (10 μ g/ml) was added to one of the cultures for 120 min to suppress enzyme production.

^c Enzyme synthesized.

passed through a gel filtration column (Fig. 7), it was demonstrated that in both cases the enzyme activity was present in the previously described (9) high-molecular-weight aggregates. The activity in the peaks from the columns contained enzyme capable of synthesizing both water-insoluble and water-soluble product from sucrose.

DISCUSSION

Attempts to purify large quantities of low-molecular-weight extracellular dextranucrase from *S. mutans* 6715 have been complicated due to the inclusion of essentially all of the enzyme activity in high-molecular-weight extracellular aggregates (9). This problem and the findings that dextranucrase activity can be affected by the presence of glucans (8), detergents (22, 23), phospholipids (12, 19, 21), and a variety of other molecules (4) indicate that the enzyme may have an unusual composition. Studies with extracellular enzymes produced by other gram-positive bacteria indicate that there may be structural characteristics which are common to enzymes

which must pass through the bacterial membrane and cell wall. A well-studied example is the extracellular penicillinase produced by *B. licheniformis* (15, 24, 25). This enzyme appears to exist as a phospholipoprotein at one time during its production. Analysis of the structure and mechanism of penicillinase formation and secretion has led to proposals as to how the unique amino acid sequences and phosphatidylserine residue may facilitate its production by the bacterium (14). The possibility that other extracellular enzymes may contain phosphate and lipid has been discussed (14). Based on the aggregating characteristics of the *S. mutans* dextranucrase, it is not unlikely that this enzyme is associated with lipid moieties at some time during its synthesis and/or secretion. Association with lipoteichoic acid has previously been reported (C. Schachte, S. Harlander, and G. Germaine, J. Dent. Res. 56A:360, 1977) and lipid has been detected in partially purified enzyme preparations using both chemical assays and radioactive precursor labeling (C. Schachte, in preparation). Pure enzyme will be required to confirm and determine the type of association with lipid and to evaluate the role of this type of component in enzyme activity and function.

As demonstrated in this manuscript the production of extracellular dextranucrase by *S.*

FIG. 7. Bio-Gel A 1.5 M chromatography of ammonium sulfate-precipitated and dialyzed dextranucrase from cells grown in the absence (A) and presence (B) of cerulenin. V_0 refers to the column void volume, and the cross-hatched area indicates fractions that turned turbid during the assay due to the production of water-insoluble product (9).

mutans is sensitive to cerulenin (Fig. 2B and 6) under conditions in which protein synthesis is relatively unaffected (Fig. 4) and lipid synthesis is markedly suppressed (Fig. 5). These data demonstrate that production of the *S. mutans* extracellular enzyme may have features in common with the previously mentioned *Bacillus* enzymes (3, 7, 15, 25). Our results on the effect of cerulenin on *S. mutans* growth and metabolism are consistent with those previously reported for *S. faecalis* (2). In these bacteria the antibiotic does not have an immediate effect on culture turbidity increase, DNA, RNA, or protein synthesis, but can cause a selective inhibition of lipid production. The two streptococcal species are almost equally sensitive to cerulenin when the antibiotic is present at relatively low concentrations (1 to 5 µg per ml).

The addition of phospholipids to partially purified dextranucrase has been shown to enhance the ability of the enzyme to polymerize the glucosyl moiety of sucrose (12, 19, 21). This suggested that the decrease in enzyme production observed in the presence of cerulenin reflected a reduced availability of excreted phospholipid essential for maximum enzyme activity. Since our water-insoluble, glucan-producing form of the dextranucrase (9) showed a greater response to the presence of phospholipid than did the water-soluble, glucan-producing enzyme (12), it was possible that the former enzyme activity was being produced in an altered form. Although the total dextranucrase activity produced in the presence of cerulenin could be stimulated by the addition of phospholipid (1.6-fold increase in enzyme activity, Table 2), the stimulation was not significantly different from that obtained with the control enzyme (1.7-fold). In additional studies we have utilized assays which allow separation of the water-soluble and water-insoluble, glucan-synthesizing activities (9) and shown that the water-insoluble product-forming enzyme is present in normal quantities (data not shown). This can be confirmed by comparing the cross-hatched areas under the enzyme activity peaks illustrated in Fig. 7. In this study we attempted to determine whether the enzyme produced in the presence of cerulenin might be present in a partially disaggregated form due to the lack of lipid production. It is clear that the enzyme produced in the presence of the antibiotic is as highly aggregated as the enzyme produced under normal growth conditions.

Our current results support the concept that the dextranucrase synthesized and excreted by *S. mutans* 6715 has features in common with extracellular enzymes produced by other gram-

positive bacteria. The mechanism of enzyme production is in some manner related to the availability of lipid. An appreciation of this association should aid in attempts to purify the different forms of the *S. mutans* dextranucrase and to evaluate the mechanism of complex glucan formation by this bacterium.

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