Sequence of Biosynthesis of the Components of the Polyene Macrolides Candidin and Candihexin: Macrolide Aglycones as Intracellular Components

JUAN F. MARTIN* and LLOYD E. McDaniel

Waksman Institute of Microbiology, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey 08903

Received for publication 25 October 1974

A sequential formation of the single components of the polyene macrolide candidin complex (heptaene) has been found. In addition to the three components occurring in the candidin complex at the end of the fermentation, two other “early” all-trans heptaene components have been characterized. They exist only during the phase of active biosynthesis of candidin. Two of the components of the polyene macrolide candihexin complex (hexaene) that have been described as lacking amino sugar were the only intracellular (mycelium-associated) components observed under conditions in which no extracellular polyene remained attached to the producing cell. The results indicate that glycosylation of the macrolide ring takes place during the secretion process.

Polypeyne macrolides are highly active antifungal antibiotics (9). They have assumed increased importance with the observation that oral administration of the heptaene candidin reduced the serum cholesterol level (24) and improved benign prostatic hypertrophy (8, 11, 20). Polypeyne macrolides are secondary metabolites produced by nongrowing cells of Streptomyces as families of closely related components (17). Individual components of polypeyne macrolide complexes exhibit a similar hydrophobic polypeyne chromophore but differ in the number of hydroxyl or methyl groups existing in the hydrophilic portion of the amphipatic macrolactone ring (9, 22). Single components of polypeyne macrolide complexes differ in their biological activities (1, 25). The heptaene candidin complex has been described as a mixture of three components, but the differences among them are unknown (3). We have shown that the hexaene candihexin complex is produced as a mixture of eight components, two of them lacking the amino sugar (mycosamine) moiety characteristic of most polypeyenes (J. F. Martin and L. E. McDaniel, submitted for publication). These candihexin components lacking the functional mycosamine moiety are inactive. The lack of amino sugar moiety in some of the components suggested a possible interconversion of components by glycosylation. Although the origins of the macrolactone ring (2, 16) and the aromatic moiety (12, 13) of some polypeyne macrolides have been elucidated, the mechanisms controlling the biosynthesis and secretion of the several components of polypeyne macrolide complexes have not been studied.

We report here a study of the sequence of formation of individual components of the candidin and candihexin complexes and their intracellular or extracellular location. A correlation of the sequence of changes in the components with other biochemical parameters of the fermentation that will be helpful in understanding the biosynthesis and secretion of the large amount of polyene secreted by the producer strains has been established (17).

**MATERIALS AND METHODS**

**Organisms.** The yellow candidin producer Streptomyces viridoflavus IMRU 3685 (27) and a colorless mutant of this strain, S. viridoflavus IMRU 3961 (formerly described as 18A2), which overproduces the hexaene candihexin complex (18), were used to study the biogenesis of the candidin and candihexin complexes. They were maintained as frozen vegetative cultures in liquid nitrogen (14).

**Fermentation.** Experiments were run in 300-ml triple-baffled flasks capped with gauze pads using 50 ml of medium (15). Inoculum development and incubation conditions were as previously reported (17). Two different complex media, (i) soybean meal-glucose (SBM) and (ii) soya peptone-glucose (SP), which support optimal polyene production were used for each strain. Sources and concentrations of nutrients have been described (17). Residual glucose was

---

1 Present address: Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass. 02139.
assayed enzymatically by the glucose oxidase method.

**Assay of polyene macrolide concentrations.** A volume of fermentation broth was diluted fivefold and extracted with an equal volume of n-butanol. Polyene concentrations in diluted butanol extracts were determined using a Cary 14M spectrophotometer (Applied Physics Corp.). Candidin and candihexin were calculated on the basis of $E_{1cm}^1$ values of 1,600 and 1,000, respectively, for the pure polyenes at 380 nm (3, 18).

**Separation and extraction of mycelium-associated and extracellular polyene macrolides.** Three flasks were removed at 8- to 12-h intervals during the fermentation, and their contents were centrifuged at 15,000 × g for 15 min. The pellets were washed twice with, and finally suspended in, 50 ml of distilled water. Both the supernatant and the suspended mycelial pellet fractions were adjusted to pH 6.5 and extracted with 50 ml of n-butanol each for 30 min. The polyene-containing upper phase was concentrated under vacuum to a final polyene concentration of 1 to 5 mg/ml for thin-layer chromatography (TLC) analysis. Polyene solutions were protected from light and refrigerated during the extraction and concentration processes to avoid polyene degradation.

**Resolution and quantitation of the components of polyene macrolide complexes.** The components of candidin and candihexin polyene complexes (1 to 5 µg applied in 1 µl) were resolved on precoated Silica Gel G 250-µm plates (Analtech, Inc.) scored in 1-cm wide strips, using the lower phase of the solvent system chloroform-methanol-20% ammonium hydroxide (CMA), 2:2:1, (vol/vol/vol). Components were visualized under ultraviolet (UV) light. Identification of the single components was done using pure isolated standards. Quantitation of the components was carried out by in situ transmission thin-layer spectrodensitometry using a Schoeffel SD-3000 double beam densitometer equipped with a SDC 300 density computer and recorder (Schoeffel Instruments Corp.). The quantitation method is being published in detail elsewhere (18a).

**Isolation and characterization of single components of polyene macrolide complexes.** The acetone-soluble, mycosamine-lacking inactive components of the candihexin complex, as well as the acetone-soluble components D and E of the candidin complex, were separated by acetone fractionation as described previously (18). Single components of candidin and candihexin complexes were isolated by preparative TLC, using 1,000-µm precoated Silica Gel G plates in the CMA solvent system. Polyene-containing bands were scraped off and the components were eluted with methanol. The extracts were rechromatographed until complete purity, as indicated by a single peak in the densitometer, was achieved. Solutions of individual components were characterized by their UV-visible absorption spectra.

**RESULTS**

**Production and secretion of candidin complex.** The pattern of the candidin fermentation parameters in SBM medium is depicted in Fig. 1a. Candidin was rapidly synthesized during a 36-h period following growth. Production stopped suddenly after glucose exhaustion. About two-thirds of the total candidin appeared as mycelium-associated product during the production period. It was later released into the medium after synthesis had ceased, to a final level of 50% of the total in the extracellular medium. The candidin production in SP medium was faster than in SBM medium but the antibiotic yield was lower. The pattern of fermentation parameters and the distribution of candidin between mycelium-associated and released fractions were similar to those obtained in SBM medium.

**FIG. 1.** (a) Production and secretion of the candidin complex (SBM medium). Symbols: Total candidin (Δ); mycelium-associated candidin (○); released candidin (Ο); glucose (■); pH (without symbols). (b) Changes in the proportions of the single components of the released (extracellular) candihexin complex. Symbols: Candidin A (Θ); candidin B (Δ); candidin D (Ξ); candin E (Ψ).
Time sequence of formation of the components of the candidin complex. The time sequence of formation of the candidin components was studied separately for the mycelium-associated and released antibiotic. Figure 2 shows the components existing in the mycelium-associated SBM candidin complex at different times during the fermentation. Soon after the beginning of the idiophase, when a low concentration of candidin had been produced, the candidin complex was composed of five components, all of them having the characteristic all-trans UV-visible absorption spectra of polyenes. They showed \( R_f \) values of 0.27, 0.30, 0.33, 0.37, and 0.40 in the CMA solvent system. They will be referred to hereafter as candidins A, B, C, D, and E (Fig. 2). Extracellular mycelium-released candidin complex showed similar composition.

Candidins D and E, which form 14 and 11%, respectively, of the candidin complex at 48 h of fermentation, do not exist in the mycelium-associated candidin complex after 72 h when production had stopped (Fig. 2). A similar decrease in the proportion of candidin D and the loss of candidin E occurred in the extracellular mycelium-released complex, coinciding with the phase of rapid candidin synthesis (Fig. 1b). After candidin biosynthesis ceased, no significant change took place in the proportion of the components in the candidin complex. The final product of the candidin fermentation in SBM medium is formed largely of candidins A and B.

The same components appeared in the candidin complex obtained in SP medium, although their proportions were quite different in the two media (Table 1). The sequence of changes in the proportion of the components of the candidin complex in SP fermentations was similar to that in SBM medium. Since candidin components D and E occurred only in the early stages of the fermentations in both SBM and SP media, they will be referred to as “early” candidin components.

Characterization of candidin early components D and E. The early candidin components D and E showed higher \( R_f \) values in the CMA solvent system than did the candidin major components A and B. They were soluble in 80% acetone, in contrast to the main candidin components which precipitate in this solvent. In both of these properties their behavior is similar to those of the mycosamine-lacking inactive components of the candihexin complex. TLC-purified candidins D and E had multipeak UV-visible absorption spectra with peaks in methanol at 405, 382, and 363 nm, identical to those of the main components A and B (Fig. 3). The sharp fine structure of the spectrum with a similar peak spacing to that of the all-trans disubstituted heptaenes and the highest peak at the longest wavelength indicated a disubstituted all-trans chromophore of seven alternating double bonds (21). The identity of the UV-visible absorption spectra of all the candidin components suggests that differences between the early candidins D and E and the main candidin components A and B are not due to different heptaene hydrophobic chromophores but to the substituents of the hydrophilic region of the macrolactone ring.

Production and secretion of the candihexin complex. The kinetics of the production of candihexin complex in SBM medium are indicated in Fig. 4. In contrast to the results described for candidin, 80% of the total candihexin was released into the broth from the

---

**Table 1. Composition of the polyene macrolide candidin complex obtained from different sources**

<table>
<thead>
<tr>
<th>Candidin Components</th>
<th>Mycelium-associated candidin from SBM medium</th>
<th>Mycelium-associated candidin from SP medium</th>
<th>Data of Borowski et al.*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 h</td>
<td>20 h</td>
<td>70 h</td>
</tr>
<tr>
<td>Candidin A</td>
<td>29*</td>
<td>64</td>
<td>71</td>
</tr>
<tr>
<td>Candidin B</td>
<td>40</td>
<td>19</td>
<td>24</td>
</tr>
<tr>
<td>Candidin C</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Candidin D</td>
<td>14</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Candidin E</td>
<td>11</td>
<td>Traces</td>
<td>0</td>
</tr>
</tbody>
</table>

*Undescribed source in reference 3.

**Fig. 2.** Sequence of changes in the proportion of the single components of the mycelium-associated candidin complex (SBM medium). Each panel shows a densitometric scanning of a TLC plate of the candidin complex extracted at a different time during the fermentation.
dihexin fermentation, with more than 90% of the candihexin being released from the mycelium. The large release of candihexin by the mutant in both SBM and SP media in contrast to the retention of candidin by the parent strain suggest that it is a characteristic property of the 18 A2 mutant acquired during the mutation.

Time sequence of formation of the components of the candihexin complex. Six components occurred in the candihexin complex (Fig. 5). Early in the idiophase the candihexin complex had a small proportion of the active component A and a large concentration of the inactive mycosamine-lacking candihexins E and F (Fig. 5). At 116 h, when antibiotic titers were maximum, the proportion of candihexin A had increased resulting in an overall increase in the bioactivity of the candihexin complex. Thereafter no significant change in the proportions of the components occurred, although a considerable reduction in antibiotic titers due to an unspecific degradation of the complex was found (Fig. 4).

Intracellular nature of the mycosamine-lacking candihexins E and F. The candihexin complex extracted from SP broth had the same components as that produced in SBM medium, but the proportions of the components were different (cf. Fig. 5 and 6). A relevant result for understanding the secretion process was the difference observed in SP medium between the components existing in the mycelium-associated candihexin complex and that released into the broth. All the components of the beginning of the production phase. The SP medium supported a fast but low-yielding candi-
and candidin complex were found in the candidinhexin complex obtained in the supernatant broth (Fig. 6), but only two of them, the mycosamine-lacking inactive components E and F, were found in the intracellular candidinhexin complex (Fig. 7). This strikingly different distribution was found in all samples taken during the fermentation. Since in this medium all the secreted candidinhexin appears released from the mycelium (more than 90% of the total candidin), the mycelium-associated complex composed of inactive mycosamine-lacking candidinhexin components seems to be truly intracellular.

**DISCUSSION**

The term mycelium-associated antibiotic refers to both intracellular and cell wall-bound antibiotic which is collected with the mycelium pellet by centrifugation. The large fraction of mycelium-associated candidin suggests that a considerable amount of candidin remains attached to the cell membrane or adsorbed onto the cell wall after secretion. The binding affinity of polyene macrolides to membrane-occurring lecithin in absence of sterols is now a well established fact (5, 25, 28, 29). Binding to lecithin occurs at polyene concentrations similar to those attained in the fermentation broth. A nonspecific binding of antibiotics to mycelium of several species of *Streptomyces* has also been reported (23). Some of the mycelium-associated candidin is truly extracellular since it can be released as polyene micelles by repeated washings with distilled water or saline solution. The binding of this large amount of candidin to the cell obscured possible differences in composition between intracellular and extracellular candidin complexes. In contrast, the mycelium-associated antibiotic in the candidinhexin fermentation (where about 90% of the candidinhexin is released from the mycelium) appears to be mostly intracellular. In this case, significant differences were found between the composition of intra- and extracellular candidinhexin complexes.

The formation in the early idiophase of candidins D and E, followed by their decrease or loss later in the fermentation, suggests that these components may be precursors of the major candidins A and B. They are either diluted thereafter by the large amount of candidin complex formed or completely transformed into the final components A and B. Since the early candidins D and E exist only during the phase of active synthesis of antibiotic, they do not appear to be degradation products which are more likely to occur in the late phases of the fermentation. The final composition of the candidin complex obtained in SP medium was similar to that described by Borkowski et al. (3), which differs from the composition of the candidin complex obtained in SBM fermentations. Although no samples of the components isolated by these authors were available for comparison, they seem to correspond to candidins A, B, and C which occur in the candidin complex at the end of the fermentation.

The exclusive intracellular presence of mycosamine-lacking candidinhexins E and F in SP medium suggests an intermediate role for these mycosamine-lacking inactive components in
the biosynthesis of active candihexins A and B. Dhar and Khan (6) have proposed that several groups of antibiotics owe their origin to detoxification of metabolites that are detrimental to the producing cell. They suggest that formation of macrolide antibiotics is initiated by a need to detoxify the cell from the unusual sugars that appear in the macrolide antibiotics. Studies on the biosynthesis of erythromycin B indicated that erythronolide B, the aglycone of erythromycin B, is an intermediate in the erythromycin B biosynthesis (10, 19, 26). According to this theory, the mycosamine-lacking candihexins E and F may be the aglycones (candihexinolides) of candihexins A and B. A similar role for the early candidins D and E is probable in view of their similarity to candihexins E and F in chromatographic behavior (fast moving) and less polar character (acetone solubility).

Very little is known about the timing when, or cellular level where, binding of the amino sugar to the macrolide ring occurs. The absence of candihexins A and B from intracellular candihexin complex, which appeared to be formed exclusively by mycosamine-lacking candihexins E and F, indicates that glycosylation takes place during secretion of the polypeptide. This evidence is consistent with the detoxification theory of Dhar and Khan (6), in which the macrolide ring acts as a lipid-soluble carrier of the amino sugar. Candihexins E and F which lack the mycosamine moiety exist also in the extracellular medium, either because part of the carrier is secreted without being charged with the sugar or as a result of the hydrolysis of the glycosidic bond after secretion, as has been pointed out by Corcoran (4). Attachment of carbohydrate to intracellular proteins also plays a general role in the secretion of glycoproteins (7). Whether the mycosamine-lacking intracellular components are true precursors of the glycosidic components, as suggested, or simply the end products of a related pathway unable to be glycosylated because of some structural difference is not yet clear.

ACKNOWLEDGMENTS

This research was supported in part by N.I.H. International Postdoctoral Fellowship 1F05 TW 1870 and a Charles and Johanna Busch Postdoctoral Fellowship.

We acknowledge the suggestions of C. P. Schaffner and the help of D. P. Bonner in preparing the manuscript.

LITERATURE CITED


