Monophenol Monooxygenase and Lincomysin Biosynthesis in
*Streptomyces lincolnensis*

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Monophenol monooxygenase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase EC 1.14.18.1) was studied in melanin-positive and melanin-negative mutants of *Streptomyces lincolnensis* NCIB 9413, varying in the lincomycin synthesizing ability. The activities of laccase and tyrosine phenol lyase (EC 4.1.99.2) are absent in this organism. The monophenol monooxygenase catalyzes hydroxylation of monophenols (Km and Vmax for L-tyrosine, 2 × 10⁻⁴ M and 8.0 nmol of O₂/min per ml, respectively) at a slower rate than it dehydrogenates diphenols to o-quinones (Km and Vmax for L-3,4-dihydroxyphenylalanine, 7 × 10⁻⁵ M and 51.7 nmol of O₂/min per ml, respectively. It is inhibited by KCN, β-mercaptoethanol, ethylenediaminetetraacetate, dipropyldithioure, p-aminobenzoic acids and by some tryptophan metabolites. Changes in the activity of monophenol monooxygenase caused by mutation or by inhibitors are reflected in the synthesis of the antibiotic. Its participation in the biogenesis of the proplyhygric moiety of lincomycin is discussed.

It has been reported that phenol oxidase enzymes participate in the formation of fruiting bodies in the basidiomycete *Schizophyllum commune* (12). Recent results on the biogenesis of lincomycin suggested that these enzymes may be involved in the synthesis of lincomycin by *Streptomyces lincolnensis* (19). The isotopic experiments performed with this organism showed that the pyrroldione structure of the proplyphydric moiety of lincomycin originates from L-tyrosine. According to these data propylproline or ethylproline, the respective precursors of propyl and ethylgyric acids, are formed from L-dopa quinone or 2-carboxy-2,3-dihydro-5,6-dihydroxyindole. The latter two compounds are also intermediates of melanogenesis. This may imply that monophenol monooxygenase is the enzyme initiating both the formation of melanin and the biosynthesis of lincomycin from L-tyrosine.

In the present study, monophenol monooxygenase from *S. lincolnensis* has been characterized, and evidence has been presented on the relation of this enzymic activity and the size of tyrosine pool to melanin formation and biosynthesis of lincomycin. It has been found that mutants with lesion in this enzyme do not produce antibiotics.

A preliminary account of this work was given at the 2nd International Symposium on Recent Progress in Antibiotic Research, Warsaw, 3–5 December 1973.

MATERIALS AND METHODS

Strains and incubation conditions. The following high lincomycin-producing and nonproducing mutants isolated from the melanin-positive (mel⁺) and melanin-negative (mel⁻) strains of *S. lincolnensis* were employed: the original strain mel⁺ NCIB 9413: mel⁺ linco⁺ 11 selected from NCIB 9413: mel⁺ linco⁺ 426 and mel⁻ linco⁺ 434, the two spontaneous lincomycin-producing mutants isolated from mel⁻ linco⁻ 11: mel⁻ linco⁻ 2 and mel⁻ linco⁻ 10, the spontaneous nonproducing mutants isolated from mel⁻ linco⁻ 433. Under our experimental conditions the yields obtained with the lincomycin-producing strains were: 1, 190, 435, and 444 μg of lincomycin per ml, respectively.

The organisms were cultivated in 500-ml Erhlermayer flasks at 28 C in a medium prepared according to Lapchinaksaya et al. (11), in which soybean flour was replaced by its papain hydrolisate and the cornsteep liquor by its soluble extract.

Preparation of extracts. Cells were separated from the medium by 10-min centrifugation at 2,000 rpm in the cold. The intracellular metabolites were extracted by homogenizing the cells in a glass homogenizer with 10% HClO₄ (1:1, wt/vol). The extracts were separated from cellular debris and neutralized with 20% KOH, and the potassium perchlorate precipitate was centrifuged off. For enzymic assay, cells were washed twice with distilled water, suspended in 0.1 M buffer tria(hydroxy-methyl)aminomethane-hydrochloride, pH 7.6 (1:2, wt/vol), and homogenized with the aid of an
RESULTS

Monophenol monoxygenase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) was found in the mel+ and mel- mutants of lincolnensis NCIB 9413. This enzyme catalyzes both orthoxygenation of monophenols and dehydrogenation of diphenols to o-quinones. The sonic extracts of S. lincolnensis do not oxidase potassium ferrocyanide and characteristic red pigmentation is not formed with p-cresol, thus indicating the absence of laccase activity in this organism. The presence of tyrosine phenol lyase (EC 4.1.99.2) may also be excluded since pyruvate could not be detected by the enzyme assay with lactate dehydrogenase (EC 1.1.1.27) in the deproteinized concentrated mixtures after 30 min of incubation with tyrosine at 30 C (10).

In S. lincolnensis, 90% of monophenol monoxygenase is found in the culture medium and only 10% is cell bound. Similar excretion of phenol oxidase enzymes is observed in fungi (15). The S. lincolnensis enzyme catalyzes the oxidation of L-tyrosine, L-DOPA, catechol, and p-cresol. The d-isomer of tyrosine is not oxidized, in contrast to the enzymes from Actinomycetes albocarotinus, A. juglandis, and A. galbus, which utilize the d-isomer as a sole source of carbon and nitrogen, although to a smaller extent than the L-isomer (9). The affinity of the S. lincolnensis enzyme towards its substrates has been expressed in Km values calculated from Lineweaver-Burke plots in Table 1; as can be seen, the Km value for tyrosine is about three times higher than for DOPA. Catechol acts as a much poorer substrate, whereas the presence of ascorbate is a prerequisite for the oxidation of p-cresol.

The activity of monophenol monoxygenase from S. lincolnensis is arrested by a variety of Cu2+ chelating compounds, tyrosine analogues, and tryptophan metabolites (Table 2). The most potent inhibition is exhibited by KCN which, at a concentration of 5 x 10-4 M, causes a 50% reduction of the oxidative activity. The same effect is exerted by α-mercaptoethanol at a concentration lower by one order of magnitude. Thiourea, EDTA, dipiridyl, and o-phenanthroline are less effective by four orders of magnitude as compared with KCN. p-Aminobenzoic acid...
Acid acts as a stronger inhibitor than the compounds of the latter group, but poorer than \( \beta \)-mercaptoethanol. Tyrosine hydroxamate is an effective inhibitor of monophenol monooxygenase, as are also some tryptophan metabolites, e.g., quinolic and nicotinic acids. Tryptophan itself is ineffective but, after 30 min of incubation of the crude extract with this amino acid, a fourfold decrease in the activity of the oxidase is observed. This is consistent with the supposition that tryptophan metabolites inhibit the activity of this enzyme and represents a known effect of intermediates of one pathway on the enzymes of another pathway branching from the same precursor-prephenate. The effect of tryptophan metabolites on phenol oxidase of human melanoma was found some years ago (16).

Monophenol monooxygenase in the biosynthesis of lincomycin. The relation of monophenol monooxygenase to the formation of the antibiotic has been studied with \( \text{mel}^+ \) and \( \text{mel}^- \) spontaneous mutants of \( S. \text{lincolnensis} \) NCIB 9413. The mutants show different lincomycin-synthesizing abilities. Lincomycin is not formed during the logarithmic phase of growth of \( S. \text{lincolnensis} \), i.e., under the applied conditions up to 24 h of cultivation (Fig. 1). In the \( \text{mel}^+ \) strain the maximum activity of the oxidase, both in the mycelium and in the medium, is observed in the 15-h cultures before synthesis of antibiotic starts. At the later stage of growth, enzymic activity rapidly decreases and in 24 h is reduced to 15% of the maximum activity in the mycelium and 30% in the medium (Fig. 2). The rapid drop in the oxidase activity is followed by a further gradual decrease in the stationary growth phase. It has been shown that the activity of the oxidase during exponential growth is not correlated with the lincomycin-synthesizing ability of the \( S. \text{lincolnensis} \) strains correlation coefficient, \( -0.16 \); unpublished data).

This lack of correlation between melanogenesis and antibiotic-synthesizing ability has been confirmed by isolation of the two high lincomycin-producing spontaneous \( \text{mel}^- \) mutants, \( \text{mel}^-426 \) and \( \text{mel}^-434 \). In both of these mutants, the activity of monophenol monooxygenase in the logarithmic phase of growth does not exceed 5% of that found in the \( \text{mel}^+ \) strains. However, in the stationary phase in which lincomycin is produced, the oxidase activity in mycelium is four to five times higher in the \( \text{mel}^-426 \) and \( \text{mel}^-434 \) mutants as compared with their parent \( \text{mel}^+434 \) strain (Fig. 2). The yield of antibiotic produced by these \( \text{mel}^-426 \) and \( \text{mel}^-434 \) mutants is about twice as high as that of the parent strain. The nonproducing mutants, \( \text{mel}^-2 \) and \( \text{mel}^-10 \), were found to be deficient in monophenol monooxygenase.

Additional evidence for a link between lincomycin synthesis and phenol oxidase was provided by experiments on the effect of inhibitors of this enzymic activity on production of the antibiotic.

Thiourea added to the culture medium before inoculation at a concentration of 10 mM does not affect growth of the parent strain but it decreases by half the oxidase activity in the logarithmic growth phase of \( \text{mel}^+11 \) strain and about 15 times in the stationary phase of growth. The yield of antibiotic is re-
duced by 90%. Formation of the antibiotic is also markedly reduced upon addition of tryptophan and its metabolites at a concentration of 10 mM to the nongrowing 48- or 72-h cultures (Table 3). Indol appears to be the most potent inhibitor of lincomycin synthesis. Introduction of this compound to the 72-h cultures results in a decrease of lincomycin yield to 30% of that produced by control mycelium without supplements. Tryptophan, anthranilic, and indolylpyruvic acids added to the 48-h cultures diminish production of the antibiotic by 60 to 70%. The same extent of inhibition is caused by indolylpyruvic acid when this compound is introduced as late as 24 h before harvesting of the antibiotic.

It seems also of interest that anthranilic acid is less effective than its 3-hydroxy derivative. Data in Table 4 show that inhibition of monophenol monooxygenase by tryptophan metabolites is associated with their effect on the yield of lincomycin. Formation of the antibiotic is less susceptible to the action of indole than the oxidase activity, whereas this activity and lincomycin synthesis are repressed to the same extent on addition of 3-hydroxyanthranilic acid.

The pool of tyrosine, phenols, and amino acids. To obtain more information on lincomycin precursors, the pool of tyrosine, phenols, and amino acids was determined in the mel+ and mel− strains, which differ in the activity of diphenol oxidase and the ability to synthesize lincomycin.

The level of total phenols remained practically unchanged during the growth (Fig. 3). The amino acid pool doubled in the mycelium at the end of cultivation and in the medium a rapid decrease in cultures was followed by their accumulation in the stationary phase of growth (Fig. 4). A low level of monophenol monooxygenase activity in the logarithmic phase of growth and the lack of melanin formation in the mel− mutants result in an accumulation of tyrosine in the culture medium (Fig. 5). In the mel−linco−2 and mel−linco−10 mutants, which do not produce the antibiotic, tyrosine forms about 10% of

<table>
<thead>
<tr>
<th>Addition*</th>
<th>Antibiotic yield% for metabolite added to the:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48-h cultures</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>33</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>35</td>
</tr>
<tr>
<td>3-Hydroxyanthranilic acid</td>
<td>30</td>
</tr>
<tr>
<td>Indolylpyruvate acid</td>
<td>41</td>
</tr>
<tr>
<td>Indole</td>
<td>26</td>
</tr>
</tbody>
</table>

* At a concentration of 10 mM.

** The antibiotic yield was determined in 96-h cultures.

![Fig. 2. Time course of monophenol monooxygenase activity in the S. lincnensis strains: mel+linco*11 (○), mel−linco*434 (△), and mel−linco*426 (□).](http://aac.asm.org/Download)
**TABLE 4. Effect of indole and 3-hydroxyanthranilic acid on the activity of monophenol monooxygenase and lincomycin synthesis in the mel-linco**

<table>
<thead>
<tr>
<th>Addition*</th>
<th>Monophenol monooxygenase activity (%)</th>
<th>Lincomycin yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Indole (2.5 mM)</td>
<td>39</td>
<td>72</td>
</tr>
<tr>
<td>Indole (5 mM)</td>
<td>23</td>
<td>43</td>
</tr>
<tr>
<td>Indole (10 mM)</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>3-Hydroxyanthranilic acid (6 mM)</td>
<td>90</td>
<td>102</td>
</tr>
<tr>
<td>3-Hydroxyanthranilic acid (10 mM)</td>
<td>66</td>
<td>63</td>
</tr>
</tbody>
</table>

* Inhibitors were added to the 42-h cultures.

**DISCUSSION**

The occurrence of monophenol monooxygenase in streptomycetes has been previously reported (7, 13). The enzyme from *S. glaucescens* has been isolated and obtained in a homogeneous state (13). In contrast to the enzyme from this organism, the oxidase of *S. lincolnensis* is extracellular, but it is not known whether it represents a single protein moiety or a mixture of multiple forms as it exists in fungi (3). The affinity for tyrosine of the *S. lincolnensis* enzymes is very similar to that of *S. glaucescens* oxidase (0.2 and 0.4 mM, respectively). However in *S. lincolnensis* the *Km* and *Vmax* values for DOPA are one order of magnitude higher than for tyrosine. The enzyme investigated is inhibited by the Cu²⁺-complexing compounds:

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**Fig. 3. Size of the phenol pool in the mel-linco**

- mel-linco²1 (□), mel-linco²426 (△), mel-linco²434 (△), mel-linco²-2 (○), and mel-linco²-10 (■). Concentration of phenols was determined by the Folin-Ciocalteu method according to Bray and Thorpe (4) in the neutralized perchlorate extracts of the mycelium (a) and the medium (b).

**Fig. 4. The amino acid pool in mycelium (A) and medium (B) of mel-linco²1 (□), mel-linco²426 (△), mel-linco²434 (△), mel-linco²-2 (○), and mel-linco²-10 (■). Concentration of amino acids was determined by the ninhydrin method of Alberti and Bartley (1) in the neutralized perchlorate extracts.**

**Fig. 5. Concentration of tyrosine in the mycelium (A) and medium (B) of the *S. lincolnensis* mutants: mel-linco²1 (□), mel-linco²426 (△), mel-linco²434 (△), mel-linco²-2 (○), mel-linco²-10 (■). Tyrosine level was measured according to Udenfriend and Cooper (18) in the neutralized perchlorate extracts.**
with KCN for DOPA oxidation is practically the same in both *Streptomyces* species (5.0 and 3.7 μM).

The results presented in this paper provide evidence for participation of phenol oxidase in the biosynthesis of lincomycin. It has been shown that the synthesis of lincomycin coincides with the activity of this enzyme in the stationary phase of growth, whereas melanogenesis is associated with this enzymic activity during exponential growth. Genetic regulation of melanogenesis in the streptomycete and its relation to the differentiation process is unknown. Some earlier results suggested the extrachromosomal control of melanin production in *S. scabies* (7) and genetic analysis of this phenomenon has been undertaken with *S. glaucescens* recently (2). The mel- mutation in *S. lincolnensis* involves the gene responsible for the expression of monophenol monooxygenase in the differentiating cell. In both mel- linco-426 and mel- linco-434 high producing mutants, the maximum activity of the oxidase is observed in the stationary phase of growth. Lack of this activity in the mel-linco-2 and mel-linco-10 mutants results in a loss of lincomycin-synthesizing ability. Suppression of this activity could be achieved by introducing inhibitors to the culture medium at the time of growth cessation. Although none of the inhibitors applied is specific for monophenol monooxygenase, they represent different types of inhibitors, and the existence of the same response of lincomycin synthesis to these inhibitors implies participation of this enzyme in formation of lincomycin. Thus, both the direct and indirect evidence presented is consistent with the proposed origin of the propylhygric moiety of lincomycin from tyrosine (9).

**LITERATURE CITED**


