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DEMONSTRATION OF A C-METHYLATING ENZYME IN CELL FREE EXTRACTS OF INDOLMYCIN-PRODUCING STREPTOMYCES GRISEUS

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Summary

A cell free extract of an indolmycin-producing strain of Streptomyces griseus is shown to catalyze the transfer of a methyl group from S-adenosylmethionine-(methyl-^{14}C) to indolepyruvate to form radioactive β-methylindolepyruvate, an intermediate in indolmycin biosynthesis. The reaction product β-methylindolepyruvate has been unequivocally identified by chemical correlations and it has been shown to have the S configuration.

We have shown that the biosynthesis of the antibiotic indolmycin (1) (I) by Streptomyces griseus involves a C-methylation reaction in which the intact methyl group of methionine is transferred to the β-carbon atom of the side chain of a tryptophan-derived compound (2,3). We wish to report here on a cell-free system from S. griseus which catalyzes the transfer of the S-methyl group of S-adenosylmethionine to indolepyruvate to form (S)-β methylindolepyruvate [(3S)-2-oxo-3-(3'-indolyl)-butyric acid] (II). Experiments are also reported which show that (S)-β-methylindolepyruvate is efficiently and specfically converted into indolmycin by intact cells of S. griseus.

Materials and Methods

Cells of S. griseus, ATCC 12648 (4), grown for 48 hours at 24° in a medium consisting of peptone (20.0 g/l), yeast extract (2.0 g/l), $FeSO_4 \cdot 7\ H_2O$ (1 mg/l) and trace element solution (5) in distilled water, pH 7, were harvested by fil-

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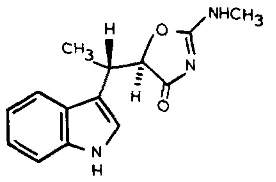
tration. The washed mycelium (1.5 g wet weight per 100 ml flask) was suspended in 10 ml per g of 10^{-2} M phosphate buffer, pH 7.0, and the cells were disrupted by one passage through a French pressure cell at 20,000 psi maximum pressure. Cell debris were removed by centrifugation at 30,000 x g for 15 minutes and the supernatant was dialyzed for 1 hour each against three changes of one liter of the same buffer. These operations were carried out at 4^{o}. The protein concentration obtained was usually 13 mg/ml as determined by the biuret method (6). Centrifugation at 105,000 x g was performed in a Beckman L2-65B ultracentrifuge for 1 hour at 4^{o}. The incubations were carried out as described in table 1.

The enzyme reaction product was cochromatographed with authentic β-methylindolepyruvate on silica gel G in acetone/chloroform/acetic acid 5:4:1 (R_F0.33) and ethyl acetate/isopropanol/acetic acid 5:4:1 (R_F 0.59). Authentic (R)-β-methylindolepyruvate was prepared from β-methyltryptophan isomer B (11,12) by reaction with L-amino acid oxidase in the presence of excess catalase. Its ORD spectrum showed a positive Cotton effect and a molecular rotation $[M]_{387m\mu} = +2780$.

For further identification of the enzyme reaction product, a larger incubation with 20 μCi of S-adenosylmethionine-(methyl-^{14}C) was carried out which produced $1.27 \cdot 10^{6}$ dpm of crude β-methylindolepyruvate. The radioactive material was dissolved in water, adjusted to pH 7 with N/50 NaOH and an aliquot was reduced with sodium borohydride to give the diastereomeric radioactive indolmycenic acids (1) (III), which after decomposition of excess $NaBH_4$ with tartaric acid were extracted into ethyl acetate. Aliquots of this extract were esterified with diazomethane and cochromatographed with authentic material on silica gel G plates (system A: ligroin (boiling range $100-115^{o}$)/isopropanol 9:1, run four times, R_F α-indolmycenic acid methyl ester 0.32, β-indolmycenic acid methyl ester 0.38; system B: chloroform/ethanol 5:1, R_F α- and β-indolmycenic acid methyl ester 0.55). The remainder of the radioactive indolmycenic acids was cocrystallized from dichloroethane with 500 mg of synthetic (7) carrier indolmycenic acid to constant specific radioactivity. The pure compound was diluted with a further quantity of carrier material and subjected to a Kuhn-Roth oxidation (8).



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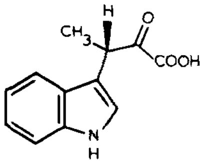
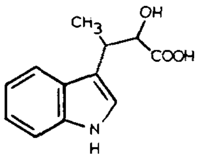


II



III

To another fraction of $7.6\cdot10^5$ dpm of sodium β-methylindolepyruvate, 150 μmoles of 3% H_2O_2 and 100 μmoles of carrier indolepyruvic acid were added. After 3 minutes at room temperature the resulting indoleisopropionic acid was extracted with ethyl acetate and the extract was chromatographed on thick layers of silica gel G in ligroin (boiling range 100-115°)/n-octanol/acetone 5:5:1 (R_F0.63, indoleacetic acid R_F0.37) to yield after elution with ether $1.34\cdot10^5$dpm of radiochemically pure indoleisopropionic acid. A part of this material ($4.06\cdot10^4$ dpm) and 200 mg of the cinchonine salt of (S)-(-)-indoleisopropionic acid (9,10) were cocrystallized from isopropanol to constant specific radioactivity.

A third fraction of $2.2\cdot10^5$ dpm of the sodium β-methylindolepyruvate was used in a feeding experiment with the indolmycin-producing S. griseus as described previously (2,3). An aliquot ($7.32\cdot10^2$ dpm) of the resulting purified indolmycin was subjected to Kuhn-Roth oxidation in the presence of carrier indolmycenic acid (100 mg = 0.457 mmoles) to give acetic acid of a specific radioactivity of $1.63\cdot10^3$ dpm/mmole.

Results and Discussion

Indolepyruvate : S-adenosylmethionine methyl transferase activity was detected in cell-free extracts of S. griseus prepared by disruption in a French pressure cell, but not in sonicated extracts. As shown in table 1, the enzyme is apparently soluble and the reaction is dependent on indolepyruvate, which cannot be replaced by L-tryptophan. The specific activity of the crude enzyme is 0.023 mμmoles per min per mg protein, which is compatible with the usual rate of in vivo production of indolmycin of about 2 mg per culture in 24 hours (1) in the same medium. The identification of the reaction product as (S)-β-methylindolepyruvate rests firmly on the following evidence:

Table 1. Conversion of indolepyruvate and S-adenosylmethionine-(methyl-^{14}C) into β-methylindolepyruvate by cell-free extracts of S. griseus.

Incubation	% conversion
Complete	6.5%
-indolepyruvate	0.5%
-enzyme	<0.1%
-indolepyruvate + L-tryptophan	0.6%
105,000 x g supernatant	7.3%
105,000 x g pellet	0.2%

The incubation mixture consisted of 1 μCi of S-adenosylmethionine-(methyl-^{14}C) (spec. act. 45 uCi/umole), 1 μmole of non-labeled S-adenosylmethionine, 1 μmole of indolepyruvate, and 1 ml of enzyme (13 mg of protein) in a total volume of 1.2 ml. After incubation for 2 hours at 30°, tartaric acid was added to give a pH of 3-4 and the mixture was extracted with ethyl acetate in the cold. An aliquot of the extract was evaporated to dryness, radioactive methanol was removed by repeated addition and evaporation of carrier methanol and the radioactivity of the residue was counted. The counts obtained in a control experiment with boiled enzyme (8.2×10^3 dpm) were substracted from those measured in the incubations.

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1. The enzyme reaction product agrees in its chromatographic mobility with authentic β-methylindolepyruvate.

2. Reduction with $NaBH_4$ gives quantitatively a mixture of radioactive α - and β-indolmycenic acid. This mixture was identified by cochromatography, as the methyl esters, with authentic indolmycenic acid methyl esters and by cocrystallization with authentic indolmycenic acids followed by a Kuhn-Roth oxidation, which gave acetic acid of identical specific radioactivity (table 2).

3. Mild oxidation with dilute hydrogen peroxide gives indoleisopropionic acid, which was identified and purified by preparative tlc. The radioactive product cocrystallized quantitatively with the cinchonine salt of authentic carrier (S)-(-)-indoleisopropionic acid (table 3). It had been found previously (13) that radioactive (R)-indoleisopropionic acid does not cocrystallize with the cinchonine salt of the (S)-isomer, 98% of the radioactivity being lost in two recrystallizations.

It was to be expected that β-methylindolepyruvate could act as a precursor of indolmycin. The correctness of this assumption was confirmed by a feeding experiment with intact cells of S. griseus, in which (S)-β-methyl-

Table 2. Cocrystallization with carrier and Kuhn-Roth oxidation of indolmycenic acid obtained from enzymatically prepared β-methylindolepyruvate.

Calculated[a]	141 dpm/mg
First crystallization	149 dpm/mg
Second crystallization	143 dpm/mg
Third crystallization	140 dpm/mg
Diluted with carrier before degradation to give	$2.17 \cdot 10^4$ dpm/mmole
Acetic acid from Kuhn-Roth oxidation	$2.11 \cdot 10^4$ dpm/mmole

[a] $7.05 \cdot 10^4$ dpm of radioactive indolmycenic acid were cocrystallized with 500 mg of carrier.

Table 3. Cocrystallization of indoleisopropionic acid obtained from enzymatically prepared β-methylindolepyruvate with the cinchonine salt of (S)-(-)-indoleisopropionic acid.

Calculated[a]	203 dpm/mg
First crystallization	191 dpm/mg
Second crystallization	191 dpm/mg
Third crystallization	191 dpm/mg

[a] $4.06 \cdot 10^4$ dpm of radioactive indoleisopropionic acid were cocrystallized with 200 mg of the salt.

indolepyruvate-(methyl-^{14}C), obtained by the methyl transferase reaction, was incorporated into indolmycin to the extent of 14.5%. Under comparable conditions, D,L-tryptophan-(β-^{14}C) gave 10.0% incorporation and (R)-β-methylindolepyruvate was practically not incorporated into indolmycin (0.3%) (3). The purified indolmycin obtained from (S)-β-methylindolepyruvate was degraded by Kuhn-Roth oxidation, using indolmycenic acid as a carrier, to give acetic acid which accounted for 102% of the specific radioactivity of the antibiotic.

To our knowledge this investigation is the first demonstration of an enzyme capable of C-methylating the β-carbon atom of an aromatic α-keto acid. Preliminary studies, to be reported later, suggest that the enzyme is not specific for indolepyruvate. The preparation at hand can, for example, also methylate phenylpyruvate and p-hydroxyphenylpyruvate, although somewhat less efficiently.

It may be noted in this connection that the C-methylating enzyme described here or a closely related enzyme could have a function in the biosynthesis of other antibiotics from Streptomycetes besides indolmycin, such as telomycin (14), which contains β-methyltryptophan, and bottromycin (15), which contains β-methylphenylalanine.

The C-methylating enzyme may be of physiological importance in the indolmycin-producing strain of S. griseus, because in all likelihood it is the first enzyme which irreversibly commits substrate to the indolmycin biosynthetic pathway. It may therefore be expected to play a major role in the regulation of this pathway.

Acknowledgements

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