FURTHER STUDIES ON THE BIOSYNTHESIS OF PYRROLNITRIN FROM TRYPTOPHAN BY PSEUDOMONAS

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Summary In the conversion of tryptophan to pyrrolnitrin, C-2 of the indole nucleus is retained, the amino nitrogen becomes the pyrrole nitrogen and the indole nitrogen gives rise to the nitro group. Tritium from C-2 of the side chain of L-, although not of D-tryptophan, is retained in the biosynthesis, suggesting that formation of the double bond at this carbon atom occurs simultaneously with or subsequent to the removal of the carboxyl group.

Pyrrolnitrin (I), an antifungal antibiotic which is produced by various strains of Pseudomonas, was isolated independently by Arima et al. (1) and at the Lilly Research Laboratories (2). Its structure was elucidated by the Japanese workers (3) and was confirmed by synthesis (4). Studies on the biosynthesis of pyrrolnitrin by Pseudomonas aureofaciens showed (2) that of a number of compounds tested only labeled D- or L-tryptophan were efficiently incorporated. Addition of tryptophan to the culture medium increased the production of the antibiotic, but only the D-isomer of the amino acid was active in this respect. 3-Chloroindole was isolated from tryptophan-supplemented cultures of the pyrrolnitrin-producing Pseudomonas strain (2), leading to the suggestion (5) that the biosynthetic sequence is initiated by a chloroperoxidase as shown in Scheme 1. This scheme received support when further work (6) demonstrated the occurrence of the amino analog of pyrrolnitrin (II) in D-tryptophan-supplemented cultures and showed its efficient conversion into pyrrolnitrin. This work also showed that D-tryptophan-^{14}C is incorporated more efficiently into pyrrolnitrin than the L-isomer. Experiments in which radioactive D- or L-tryptophan were fed in the presence

of large non-labeled pools of either isomer supported the conclusion that pyrrolnitrin biosynthesis apparently proceeds through D-tryptophan.

METHODS

Labeled precursors. D,L-Tryptophan-(alanine-3-^{14}C) was obtained from Amersham-Searle and D,L-tryptophan-(indole-2-^{14}C-1-^{15}N) was a gift from Professor E. Leete, University of Minnesota, Minneapolis. D,L-Tryptophan-(alanine-2-^{15}N) was material used in previous work (7), as was the D,L-tryptophan-(alanine-2-^{3}H) used in experiments 3 and 4, while that used in experiments 5-7 was prepared more recently by the same procedure (7). These two samples contained at least 97.6% and 95.8%, respectively, of their tritium in the position indicated, as determined by enzymatic degradation to indoleacetic acid. Doubly labeled samples were obtained by mixing the appropriate singly labeled specimens. The multiply labeled D- and L-tryptophan samples were prepared by treatment of the corresponding D,L-forms with L- or D-amino acid oxidase as described previously (7). In the case of the D-amino oxidase reaction a large hydrogen isotope effect (8) prevented complete oxidation of the tritiated D-tryptophan. Thus, after three incubations with the enzyme the material still contained an appreciable amount of tritium (2.3 dpm T per dpm ^{14}C), but practically no ^{14}C in the form of D-tryptophan. For the accurate determination of the T/^{14}C ratios, aliquots of the precursor solutions were cocrystallized with excess non-labeled carrier tryptophan of the appropriate configuration.

Feeding experiments. Strain A 10338.5 of Pseudomonas aureofaciens (2) was used in these studies and the experiments were conducted as described previously (2,6). Radioactive precursors were added 24 hours after inoculation and the cultures were harvested 5 days later, unless stated otherwise. The isolation of pyrrolnitrin followed the procedure given earlier (2,6). In the ^{15}N experiments the toluene extract of the cultures was subjected to tlc (silica gel G, benzene) followed by preparative glc (6' x 1/8" column, 3% SE 30 on Gas Chrom P, 40-60 mesh, helium 60 ml/min, column temperature 195°,

thermal conductivity detector) of the eluted pyrrolnitrin. These samples were then analyzed for their ^{15}N content by mass spectrometry in a Hitachi RMU 6A instrument (direct inlet, probe temperature 90°, ionizing voltage 75 eV), comparing the peak heights of the molecular ion and the peak at m/e=229 and their isotope satellites with those of the unlabeled material. Their specific radioactivities were determined by counting one aliquot and quantitating another using the u.v. absorption at 250 nm (ε =7500). In experiments requiring only the determination of T/^{14}C ratios, the toluene extracts were mixed with 100 mg of carrier pyrrolnitrin, which was then re-isolated by column chromatography (silica gel Woelm, benzene) and crystallization from ether-hexane to constant specific radioactivity. All radioactive samples were counted in a Beckman LS 100 scintillation counter using PPO and dimethylPOPOP in toluene as scintillator solution and methanol or water/BIO-SOLV BBS-3 (Beckman) as solvents. Counting efficiencies were determined for each sample using internal standards.

RESULTS

The results of a number of experiments with multiply labeled tryptophan samples are summarized in Table 1. Experiment 1 shows that both the indole nitrogen and carbon atom 2 of the ring of tryptophan are incorporated into pyrrolnitrin. The precursor contained 90 atom % ^{15}N excess in the indole nitrogen; analysis of the molecular ion region of pyrrolnitrin showed 29.2 atom % ^{15}N-enrichment. Determination of the ^{15}N content in m/e 229 was hindered by the presence of overlapping peaks resulting from loss of both 27 (HCN) and 28 mass units from M^+ (9). These ions were enriched in ^{15}N but calculated values were higher than comparable data from the molecular ion. Experiment 2 shows similarly that the amino nitrogen of tryptophan is incorporated. In this case, the precursor contained 53.8 atom % ^{15}N excess in the labeled nitrogen, the molecular ion in the mass spectrum of the pyrrolnitrin produced showed 4.2% ^{15}N excess. The M-27 cluster was identical to that in the spectrum of unlabeled pyrrolnitrin, indicating that virtually all the

Expt. No.	Precursor	Pyrrolnitrin formed (μmoles)	Incorporation of ^{14}C	Incorporation of ^{3}H	Incorporation of ^{15}N	Retention of ^{3}H[a]	Retention of ^{15}N[a]
1	56.8 μmoles D,L-Tryptophan-(indole-2-^{14}C-1-^{15}N), $5.17 \cdot 10^6$ dpm ^{14}C and 51.1 μatoms ^{15}N excess	18.75	10.05	—	10.7	—	107%
2	49 μmoles D,L-Tryptophan-(alanine-3-^{14}C-2-^{15}N), $1.13 \cdot 10^7$ dpm ^{14}C and 26.4 μatoms ^{15}N excess	18.35	15.6	—	2.9	—	19%
3	19.6 μmoles D,L-Tryptophan-(alanine-3-^{14}C-2-^{3}H), $4.71 \cdot 10^6$ dpm ^{14}C and $1.70 \cdot 10^7$ dpm ^{3}H	19.9	16.1	4.2	—	26%	—
4	48.2 μmoles D-Tryptophan-(alanine-3-^{14}C-2-^{3}H-2-^{15}N), $1.69 \cdot 10^6$ dpm ^{14}C, $1.03 \cdot 10^7$ dpm ^{3}H and 21.0 μatoms ^{15}N excess	19.8	18.0	0.36	1.76	2%	10%
5	14.5 μmoles D-Tryptophan-(alanine-3-^{14}C-2-^{3}H), $1.0 \cdot 10^6$ dpm ^{14}C and $6.35 \cdot 10^6$ dpm ^{3}H	n.d.	15.8	0.52	—	3.3%	—
6	14.5 μmoles D,L-Tryptophan-(alanine-3-^{14}C-2-^{3}H), $1.0 \cdot 10^6$ dpm ^{14}C and $6.40 \cdot 10^6$ dpm ^{3}H	n.d.	13.5	4.07	—	30.2%	—
7	14.5 μmoles L-Tryptophan-(alanine-3-^{14}C-2-^{3}H), $1.0 \cdot 10^6$ dpm ^{14}C and $6.32 \cdot 10^6$ dpm ^{3}H	n.d.	9.6	6.85	—	71.3%	—

[a] % Tritium- and ^{15}N-retention are defined as 100 x % incorporation of ^{3}H or ^{15}N / % incorporation of ^{14}C

n.d. = not determined

Table 1: Incorporation of multiply labeled tryptophan into pyrrolnitrin by Pseudomonas aureofaciens

excess ^{15}N of the sample was confined to the pyrrole nitrogen. Unlike in Experiment 1, the two isotopic labels were not incorporated to the same extent, but rather, only about 20% of the ^{15}N was retained during the conversion of tryptophan into pyrrolnitrin. Experiment 3 indicates that this loss of ^{15}N is paralleled by a similarly large loss of tritium from C-2 of the tryptophan side chain, suggesting that both occur in the same process, possibly by a reversible transamination or deamination of the amino acid. In view of the earlier experiments (6), which suggested that D-tryptophan is a closer precursor to pyrrolnitrin than the L-isomer, it would be expected that the retention of tritium and ^{15}N from C-2 of the side chain of D-tryptophan would be higher than with the racemate. However, just the contrary is observed (Expt. 4). The tritium in this case is lost entirely and the ^{15}N retention is only half of that from the D,L-form. By extrapolation this implies that more of the tritium and the ^{15}N is retained from the L-form than from D,L-tryptophan. For the tritium at C-2 of the side chain this was confirmed in experiments 5-7, in which the tritium retention from the D-, L- and D,L-form was compared. Clearly, D-tryptophan again was incorporated with complete loss of the tritium, L-tryptophan with predominant retention of the tritium, and the value for the D,L-form was between the two. In addition,

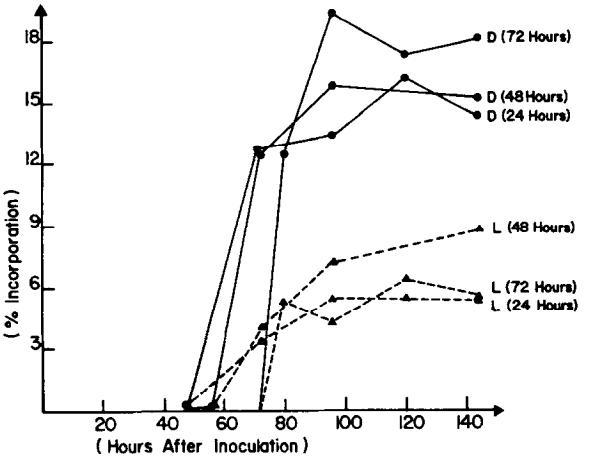
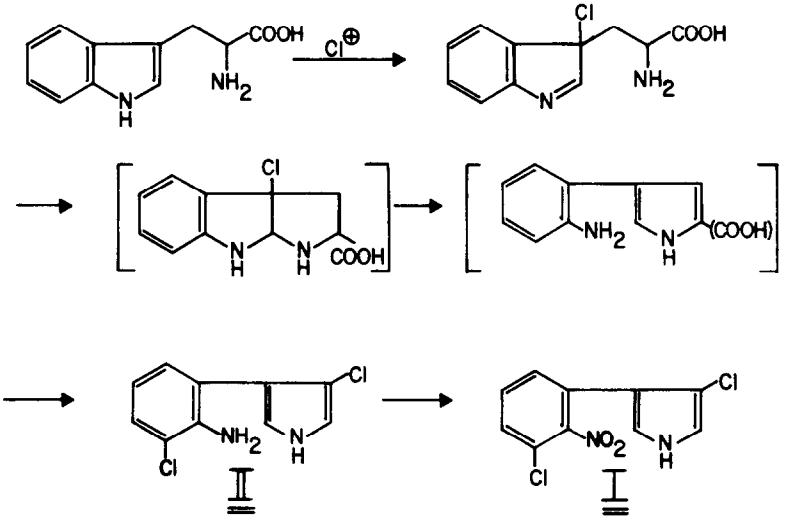


Figure 1: Incorporation of D- and L-tryptophan into pyrrolnitrin by Pseudomonas aureofaciens upon addition at different times after inoculation.

this experiment confirmed the earlier finding (6) that D-tryptophan is converted more efficiently into pyrrolnitrin than L-tryptophan. The latter point was brought out even more clearly in a time-course study (Figure 1), in which under all conditions used the D-isomer was incorporated more efficiently than the L-form.

DISCUSSION

The results support the proposed (5) pathway of pyrrolnitrin formation (cf. Scheme 1) by showing that the amino nitrogen of tryptophan becomes the pyrrole nitrogen of the antibiotic, the indole nitrogen gives rise to the nitro group, and carbon atom 2 of the indole ring is retained during the biosynthesis, presumably to become one of the carbon atoms of the pyrrole ring.



Scheme I

In addition, the retention of the tritium from C-2 of the tryptophan side chain indicates that the formation of the double bond at this carbon atom must occur simultaneously with or subsequent to the removal of the carboxyl group. At the moment we have no explanation for the surprising finding that only L- but not D-tryptophan is incorporated with retention of the tritium

from C-2 of the side chain. Together with the ^{15}N data, this observation would be best interpreted by assuming that L-tryptophan is a more immediate precursor of pyrrolnitrin than the D-isomer. However, this explanation is contradicted by the finding, in this study as well as in previous work (6), that consistently D-tryptophan is incorporated into the antibiotic more efficiently than the L-form and that it is also the only isomer effective in stimulating antibiotic production. Alternatively, the retention of tritium from C-2 of the L- but not the D-isomer could be explained by assuming that both compounds are utilized in the initial steps and are both stereospecifically decarboxylated to give an intermediate in which the carbon atom carrying the tritium has become a methylene group. Subsequent stereospecific elimination of one hydrogen during double bond formation would remove the tritium from the intermediate obtained from D-tryptophan and the unlabeled hydrogen in the other case. As a third possibility, D- and L-tryptophan might be converted into pyrrolnitrin by different routes. Additional experiments will be required to clarify this point.

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